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DEMONSTRATION OF RH ANTIBODIES IN THE NEWBORN AND FURTHER EVIDENCE OF THE PATHOGENESIS OF ERYTHROBLASTOSIS

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IT IS generally accepted that hemolytic disease of the newborn, or erythroblastosis, with a few possible exceptions is caused by the production of Rh antibodies in Rh-negative mothers bearing Rh-positive children. The present concept of the pathogenesis of this disease is based upon the reports of Levine and Stetson,[1] Landsteiner and Wiener,[2] Levine and associates,[3-5] Wiener and co-workers,[6, 7] Witebsky and Heide,[8] and others. If the concept of Forbus[9] that disease is reaction to injury is applied to erythroblastosis, it would seem desirable to attempt to establish the chain of evidence demonstrating the source and nature of the injury and the reaction to it. Although the evidence for each step in the pathogenesis of the disease is not complete, the sequence of events may be outlined as follows: (1) The Rh antigen in the fetal erythrocytes inherited from the father (2) gains access to the mother's circulation, (3) evoking a maternal response of Rh antibodies (4) which, in turn, pass across the placenta into the fetal circulation or, after birth, are absorbed from the mother's milk and (5) are adsorbed upon the infant's red cells, (6) resulting in their destruction, to produce anemia and jaundice. Further reactions on the part of the tissues of the newborn, such as myeloid hyperplasia, ectopic hematopoiesis, and edema, then follow, according to the severity of the disease. While the previously mentioned steps are commonly accepted, it should be noted that number 2, the mode of access of fetal cells to the maternal circulation, probably cannot be directly determined. However, Levine[10] has shown by analogy how minute quantities of human red cells may immunize rabbits and suggests that comparable numbers of erythrocytes might easily enter the maternal circulation through the slight placental defects known to exist. Furthermore, the production of antibodies in the mother is in itself evidence of the introduction of antigen of the type present in the fetal cells.

In this report we are concerned principally with the demonstration of evidence for events 4, 5, and 6 in the pathogenesis of erythroblastosis as outlined. This evidence consists of the identification of Rh antibodies in fetal serum, their specific adsorption on the cells of the newborn, and the hemolytic destruction of Rh-positive human erythrocytes in vitro. The demonstration of Rh antibodies in the infant has been difficult and uncertain, although Haberman and Hill[11] in 1944 first described the finding of Rh agglutinins adsorbed on the erythrocytes

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of the newborn as well as free in the serum of cord blood. The adsorbed anti-Rh agglutinins were successfully demonstrated by the elution technique of Landsteiner and Miller[12] in which the agglutinins were removed from the red cells into saline solution by brief subjection to 56° centigrade. This method of demonstrating adsorbed Rh agglutinins has recently been confirmed by Carter and Loughrey.[13] Although we have employed the elution technique routinely in all cases of hemolytic disease of the newborn since our first report, positive tests have been found in only three out of a total of ten cases of erythroblastosis. The presence of antibody adsorbed on the Rh-positive erythrocytes of the infant was never demonstrated except when also present free in the infant's serum and in the serum of the mother. The use of the blocking test slightly increased the number of positive demonstrations of fetal antibodies as shown in Case 2 (eluate) and in Case 3 (in vivo blocking).

A new approach to this problem of detecting fetal antibodies is the use of antihuman globulin serum. Coombs, Mourant, and Race[14, 15] have described the successful use of such a serum for the detection of weak and incomplete or blocking antibodies in various sera from isoimmunized individuals and suggested it might be employed to identify fetal antibodies not otherwise demonstrable. In a recent paper the same authors[16] reported on the actual use of their method to detect in vivo isosensitization of red cells in babies with hemolytic disease. The action of the antihuman globulin serum is to develop an observable agglutination where the specifically adsorbed antibodies are not able to do so by themselves (blocking or incomplete antibodies). Because of the analogy to the photographic development of the visible image, for convenience we have applied the descriptive term "developing test" to the use of the antihuman globulin serum to develop an obvious agglutination reaction. In our routine service we have employed this "developing" serum both as a test for weak antibodies in the serum of mothers and as a diagnostic test for hemolytic disease of the newborn.

METHODS

Testing of the serum of mother and infant was done, in all cases where isoimmunization was suspected, by the test tube method of Levine and Stetson.[1] Wiener's method was used to detect the incomplete or blocking antibody described by Race[17] and Wiener[18] as soon as these reports were available. The slide test of Diamond and Abelson[19] and the conglutination test of Wiener[20] were used in some studies but were not employed routinely.

Elution Test.—The elution technique of Landsteiner and Miller has been used routinely to attempt to demonstrate adsorbed Rh agglutinins on the cells of the blood of all cases of hemolytic disease of the newborn and on the erythrocytes of all babies whose mothers showed a positive test for Rh antibodies. The red corpuscles of cord blood were washed three times in cold saline, thrown down by centrifugation, and, after removing the saline the third time, 1 c.c. of fresh saline was added to a 0.25 c.c. aliquot of packed cells. After resuspension these cells were subjected to 56° C. in a water bath for five minutes. At the end of this period the suspension was immediately spun at 3,000 R.P.M. for

five minutes keeping the temperature at 56° C. with hot water in the centrifuge cups. The supernatant saline eluate was removed and recentrifuged to insure removal of all infant cells. This hemoglobin tinted fluid was then used with known Rh-positive cells by mixing one drop of the eluate with one drop of 2 per cent suspension of erythrocytes. After one hour incubation at 37° C. the tube was centrifuged gently and examined for agglutination.

Blocking Test.—If no clumping was seen in the agglutination test, one drop of anti-Rh serum of known potency was added to each tube and after a second incubation of one hour observed for agglutination. Lack of agglutination after this addition of known Rh antibody indicated presence of blocking or incomplete agglutinins.

Developing Test.—For the purpose of detecting weak Rh agglutinins, incomplete antibodies, or immune globulin, an antihuman globulin serum as described by Coombs, Mourant, and Race[15] was used. If the routine test for agglutinins using known Rh-positive cells was negative, the red cells of this suspension were washed three times with saline to remove mechanically adherent serum proteins. One drop of the developing serum was then added to one drop of the suspension of washed Rh-positive erythrocytes. Following the incubation for one hour at 37° C. the tubes were centrifuged lightly and observed for agglutination. A positive developing test indicating specifically adsorbed immune globulin was shown by definite clumping of the red cells.

Developing Test for Sensitized Erythrocytes.—As a possible diagnostic test for erythroblastosis, the developing serum was used to demonstrate the presence of antibody specifically adsorbed on the red cells in vivo. The erythrocytes of suspected cases of hemolytic disease of the newborn were washed three times to remove mechanically adherent globulin and then suspended in saline. One drop of developing serum was added to one drop of 2 per cent suspension of these washed erythrocytes and incubated for one hour at 37° centigrade. The tubes were then centrifuged at 500 R.P.M. for one minute and examined for agglutination. As a control, one drop of developing serum was added to a 2 per cent suspension of washed known Rh-positive cells previously suspended in serum containing known incomplete Rh antibodies. A second control using Rh-negative erythrocytes treated in a similar manner was used. Initially a third control consisting of a drop of washed Rh-positive cells, not previously sensitized, plus a drop of developing serum was also employed.

Demonstration of Hemolytic Activity of Rh Serum in Vitro.—To 3 c.c. of freshly drawn heparinized Rh-positive whole blood was added 0.3 c.c. of anti-Rh serum having a titer of 1:20,000 resulting in an effective final titer of 1:2,000. In order to obtain the net hemolysis due to the action solely of the Rh serum, it was necessary to set up reagent blanks and a suitable control. The first blank consisted of 0.3 c.c. of the anti-Rh serum and 3 c.c. of normal saline to make a final volume equal to that of the test and was used for the purpose of determining the amount of hemoglobin already present in the anti-Rh serum used in the experiment. The second blank was made up of 3 c.c. of saline and 0.3 c.c. of the normal serum used in the control in place of the anti-Rh serum

used in the experiment. The control set up for the purpose of determining the amount of hemoglobin released by normal heparinized blood without antiserum consisted of 3 c.c. of the same heparinized blood used in the experiment plus 0.3 c.c. of the normal serum used in the second blank.

In order to eliminate the possible hemolytic effect of mechanical action or introduction of bacterial contaminants, all the blood used in the experiment was drawn through a No. 19 needle into a dry 50 c.c. syringe and after removal of the needle gently expelled into a sterile 125 c.c. flask containing 0.2 c.c. of sterile heparin solution (1,000 units per c.c.) and gently rotated. Three cubic centimeter aliquots were immediately added to each of twelve sterile test tubes, the first six of which contained 0.3 c.c. of the 1:20,000 titer anti-Rh serum and the second, or control, series of six tubes contained only 0.3 c.c. of normal serum.

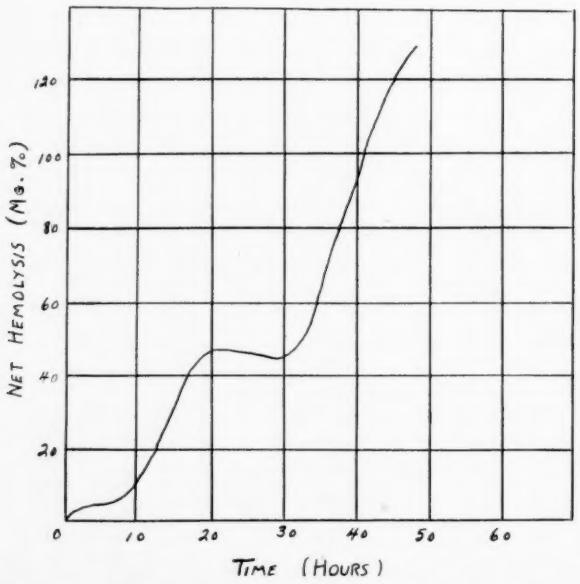

Fig. 1.—The hemolytic action of anti-Rh serum (1:2,000 titer) on heparinized Rh-positive whole blood, plotted as net hemolysis (mg. % hemoglobin) after deducting hemoglobin released in control blood not treated with anti-Rh serum.

After mixture, one tube from the experiment and one from the control series were immediately centrifuged and the supernatant cell-free fluid analyzed for the hemoglobin content by a modification of the Bing and Baker[21]* quantatative test for hemoglobin. The remaining ten tubes were incubated at 37.5° C. and one tube from each series withdrawn at 2, 6, 20, 30, and 48 hours for analysis of the hemoglobin content of the supernatant cell-free plasma. The color reactions were read in a Lumetron two-cell photoelectric colorimeter using narrow band filters and a high sensitivity double reflecting galvanometer.

*The modification consists essentially of the use of highly purified reagent grade benzidine dihydrochloride (Merck & Company, Inc., New York, N. Y.) in place of the cruder benzidine base for the benzidine reagent. The use of this product eliminates the necessity of purification with charcoal, since a blank using the dihydrochloride produces no appreciable color and if stored in an icebox, it maintains its effectiveness for a period of days.

Net hemolysis was calculated in mg. of hemoglobin from the formula $(H_t - b_t) - (H_c - b_c)$ = net hemolysis.

Where

H_t = Mg. % of hemoglobin in supernatant of tubes containing blood and antiserum (experiment).
H_c = Mg. % of hemoglobin in supernatant of tubes containing blood and normal serum.
b_t = Mg. % of hemoglobin in first blank (Rh serum + saline).
b_c = Mg. % of hemoglobin in second blank (normal serum + saline).

The results are shown in Table II and represented graphically in Fig. 1.

RESULTS

CASE 1.—O. M. was a severely icteric full-term Rh-positive male infant with enlarged liver and spleen and with erythroblasts and normoblasts in the blood. The Rh-negative mother had previously given birth to two Rh-positive children. The first was normal and still living; the second, icteric at birth, had symptoms suggestive of kernicterus and died at the age of 1 year. Before delivery the mother's serum showed Rh agglutinins with a titer of 1:20 for Rh'_o red cells and 1:40 Rh''_o cells. After delivery the mother's serum showed 1:64 and 1:256 titers for the same respective Rh-positive erythrocytes. The infant's cord serum showed positive agglutination against Rh'_o cells but not against Rh''_o. However, the eluate from the infant's erythrocytes contained antibodies which agglutinated both Rh'_o and Rh''_o. A transfusion was started one hour after birth (100 c.c.), and 50 c.c. transfusions were given daily thereafter for a week. The child is now living and well.

Diagnosis: Severe hemolytic disease of the newborn.

CASE 2.—H. R. was an icteric full-term Rh-positive male infant with erythroblasts and normoblasts in the blood film. The Rh-negative mother had two normal Rh-positive children living and well. The mother's serum after delivery showed a 1:4 titer against Rh'_o and Rh''_o erythrocytes and a blocking antibody to 1:2. The infant's cord serum showed a 1:2 titer against Rh-positive cells, but no blocking antibody was demonstrated. The eluate of the infant's red cells showed very weak agglutinins and blocking antibodies against Rh'_o and Rh''_o cells. Complete recovery followed transfusions of Rh-negative blood. The child is now living and well.

Diagnosis: Moderately severe erythroblastosis.

CASE 3.—K. D., a white, Rh-positive, female, full-term infant apparently was normal at birth and turned icteric on the second day. The Rh-negative mother had had one former child which died shortly after labor. There were no previous abortions, miscarriages, or transfusions. The mother's undiluted serum showed agglutination of Rh-positive cells and a positive blocking test at a dilution of 1:16. The cord blood was not available for elution since this was an outside case. However, the infant's red cells typed Rh negative on the first day. On the second day a weak positive reaction was obtained using the same Rh-typing serum. On the third day the child was clearly positive, thus demonstrating that in vivo blocking antibody was present on the infant's erythrocytes at birth. Blood films showed progressive increase of erythroblasts and normoblasts with a maximum of 59 erythroblasts and 12 normoblasts per 100 leucocytes on the third day. The child was taken off the breast, and transfusions were started on that day. At the age of 1 year it was reported that the child did not have normal control of muscles.

Diagnosis: Severe hemolytic disease of the newborn with kernicterus.

CASE 4.—E. K., a white, full-term, normal-appearing, Rh-positive male infant developed moderate icterus on the second day. This jaundice cleared up partially by the time the baby was discharged. The erythrocyte count was 6,100,000 per cubic millimeter the first day and 5,600,000 on the sixth. In the blood film 2 normoblasts per 100 leucocytes

were seen on the second day but none thereafter. The Rh-negative mother showed agglutinating antibodies at a titer of 1:32, blocking antibodies at 1:2, and a positive developing test at 1:32. The two previous children ages 7 and 3, were living and well. The infant showed agglutinating antibodies in the undiluted cord blood serum. No blocking antibodies were found. The developing test was not performed on the infant's blood. No transfusions were given.

Diagnosis: Mild hemolytic disease of the newborn.

CASE 5.—R. V., a white, full-term Rh-positive infant was stillborn. The mother had had four previous pregnancies: the first was stillborn prematurely; the second having erythroblastosis was transfused and is now living; and the third and fourth pregnancies terminated in abortions. After the first miscarriage the mother had received several transfusions. The mother's serum showed an agglutinating titer of 1:64 against both Rh'_o and Rh''_o erythrocytes, no blocking antibody, and a titer of 1:64 by the developing test performed months later on serum preserved by freezing. Agglutination and blocking tests on the cord serum were negative, but a positive result was obtained on the undiluted cord serum with the developing test even though the cord serum had been preserved six months by freezing.

Diagnosis: Severe hemolytic disease of the newborn with fetal death.

CASE 6.—W. C., a white Rh-positive female infant, two weeks premature, appeared normal at birth but developed mild jaundice on the second day which cleared by the time of discharge on the eighth day. Clinically this was considered to be physiologic icterus. By a former husband the Rh-negative mother had had one child which had died of pneumonia at ten months. The mother's serum showed agglutinating antibodies at 1:2 dilution, no blocking antibodies, and developing antibodies at 1:64 dilution. The infant's serum was negative to agglutinating, blocking, and developing tests for antibodies. However, the infant's washed erythrocytes gave a 4 plus agglutination with developing serum. After elution these red cells failed to clump with the same antihuman globulin serum. Nevertheless the antibodies in the eluate could not be detected even with known Rh-positive cells and developing serum. The hemoglobin was 20.15 Gm. per 100 c.c. on the third day and 14.14 Gm. on the sixth. The child was taken home on the eighth day but became progressively paler. The infant's red cells still tested 3 plus with developing serum on the nineteenth day. As a result of this serologic evidence of adsorbed hemolytic antibody still present on the erythrocytes, the baby was taken off the breast and admitted to the hospital. On the twenty-eighth day the erythrocyte count was 2,300,000, hemoglobin 6.6 Gm., and the blood film showed anisocytosis, stippling, and one normoblast. At this time the infant's washed red cells still showed a 2 plus reaction with developing serum. Two transfusions of 100 c.c. each were given, and the child was discharged in good condition. At thirty-six days the washed red cells gave a negative test with the developing serum.

Diagnosis: Mild hemolytic disease of the newborn due to Rh isoimmunization aggravated by breast feeding. Recovery with transfusion after removal from the breast.

CASE 7.—M. L., a white, male, Rh-positive full-term icteric infant had 14 per cent normoblasts and a red cell count of 4,000,000 on day of birth. The Rh-negative mother had a history of two previous pregnancies, with one child, 4 years old living and well and with one miscarriage at three months gestation since birth of the first child. No transfusions had been given. The mother's serum showed a negative test for agglutinating Rh antibodies but positive tests for blocking antibodies at 1:2 dilution and developing antibodies at 1:16. Infant's erythrocytes were not available for performance of the developing test. The baby was given a transfusion of 110 c.c. of blood on the second day which elevated the red cell count from 2,800,000 to over 3,000,000. On the fourth and twelfth days additional transfusions of about 100 c.c. each were given. No normoblasts were seen in the blood film after the third day, and the red count slowly rose to 4,200,000 on day of discharge. The infant had convulsions from the fourth day up to the twelfth day, the most severe seizure on the tenth

being accompanied with cyanosis. At last report, about one month later, the child looked well but had slightly over 2,000,000 red cells.

Diagnosis: Moderately severe hemolytic disease of the newborn.

CASE 8.—J. E. was a white, female, macerated stillborn infant, weighing 3 pounds, 11 ounces; the estimated period of gestation was eight months. The Rh-negative mother had received two transfusions following an appendectomy two years ago. There were no other pregnancies. The mother's serum showed agglutinating antibodies at 1:2 dilution, blocking antibodies at 1:32, and developing antibodies at 1:2048. Unfortunately, no specimens were obtained from the macerated edematous fetus.

Diagnosis: Hydropic, macerated fetus apparently due to Rh isoimmunization.

CASE 9.—W. S., a white, full-term, Rh-positive female infant was apparently normal at birth but turned icteric on the first day. The Rh-negative mother had one previous normal child now living and well. There was no history of transfusions or other pregnancies. The mother's serum showed an agglutinating titer of 1:128 for Rh-positive cells, a negative blocking test, and a positive developing test at a titer of 1:128. The infant's serum showed negative agglutinating and blocking tests but a positive developing test with the serum undiluted. The baby's washed erythrocytes, however, showed a 4 plus reaction with developing serum. Furthermore, the eluate from the infant's red cells showed a positive agglutinating test at 1:4 dilution and a positive developing test at 1:8. No blocking antibodies were found. On the second day after birth the erythrocyte count was 3,456,000 with 15 normoblasts and 12 erythroblasts. Seventy-five cubic centimeters of Rh-negative blood were given on the second, 60 c.c. on the fourth, and 75 c.c. on the sixth day after birth. The child continued to remain jaundiced, stools were clay colored, and there was twitching and poor coordination of muscles. There was a loss of weight from 7 pounds, 15 ounces at birth to 6 pounds, 15 ounces at the seventeenth day. The red cell count at this time was 4,650,000 with 14.4 Gm. of hemoglobin.

Diagnosis: Severe hemolytic disease of the newborn with liver damage and possible kernicterus.

CASE 10.—W. L., a white, male, Rh-positive full-term infant was normal at birth and was discharged in good condition on the fifth day. No jaundice or other significant abnormality was noted at any time. The Rh-negative mother had received several transfusions during the three weeks of hospitalization following the delivery of her first child. The mother's serum showed no Rh antibodies by any of the three tests following the delivery of this second child. However, the infant's washed erythrocytes showed definite (2 plus) agglutination with developing serum. The eluate of the infant's red cells and the cord serum failed to show antibodies with any of the three tests, agglutination, blocking, or developing. Because of the definite developing test with the Rh-positive baby's erythrocytes it was concluded that antibodies in the mother's serum too weak to be detected had passed the placenta and had become concentrated on the fetal red cells. Accordingly, the infant was taken off breast feedings.

Diagnosis: Subclinical hemolytic disease of the newborn.

A summary of the serologic findings in the ten cases described is tabulated in Table I. The severity of the erythroblastosis, treatment, and outcome are also listed.

Since the developing test applied to erythrocytes of the newborn showed promise of being diagnostic for erythroblastosis, its specificity was investigated by performing routine developing tests on the erythrocytes of all cord bloods. A total of 365 developing tests on cord cells was done. Of these all tests were negative except erythroblastosis Cases 6, 8, and 9, previously described, together with Case 10 which did not show clinical erythroblastosis but which presented a background and history compatible with a subclinical isoimmuniza-

TABLE I

	Rh antibodies in serum			Antibody adsorbed on R.B.C.	Antibody in eluate from infant R.B.C.			Severity of erythroblastosis	Outcome	Treatment
	Agglutinating	Blocking	Developing	Developing test	Agglutinating	Blocking	Developing			
Case 1								Severe	Recovery	Early transfusions
Mother	1:256	1:2	1:4†	*						
Infant	1:1	–	1:4†	*	1:4	–	1:4†			
Case 2								Moderately severe	Recovery	Early transfusions
Mother	1:4	1:2	1:16							
Infant	1:2	–	*	*	–	1:1	*			
Case 3								Severe	Kernicterus	Delayed transfusions
Mother	1:1	1:16								
Infant	*	In vivo	*	*	*	*	*			
Case 4								Mild	Recovery	No transfusions
Mother	1:32	1:2	1:32†							
Infant	1:1	–	*	*	1:1	*	*			
Case 5								Severe	Stillborn	
Mother	1:64	–	1:64†							
Infant	–	–	1:1	*	–	–	–			
Case 6								Moderate	Recovery	Off breast and transfused on nineteenth day
Mother	1:2	–	1:64							
Infant	–	–	–	++++	–	–	–			
Case 7								Moderately severe	Partial recovery; still anemic	Transfusions begun on second day; no breast feeding
Mother	–	1:2	1:16							
Infant	*	*	*	*	*	*	*			
Case 8								Severe	Macerated fetus	
Mother	1:2	1:32	1:2048							
Infant	*	*	*	*	*	*	*			
Case 9								Severe	Partial recovery; kernicterus	Delayed transfusion
Mother	1:128	–	1:128							
Infant	–	–	1:1	++++	1:4	–	1:8			
Case 10								Subclinical	Recovery	No treatment
Mother	–	–	–							
Infant	–	–	–	++	–	–	–			

*Test not done.
†Serum preserved several months in frozen state.

TABLE II.

Time (hr.)	H_t hemoglobin in Rh-treated sample (mg. %)	b_t hemoglobin in first blank (Rh serum + saline) (mg. %)	H_c hemoglobin in untreated sample (mg. %)	b_c hemoglobin in second blank (normal serum + saline) (mg. %)	$(H_t - b_t) - (H_c - b_c) =$ net hemolysis in Rh-treated sample (mg. %)
0	6.2	2.2	7.7	5.4	1.7
2	8.0	2.2	8.4	5.4	2.8
6	9.2	2.2	8.5	5.4	3.9
20	73.0	2.2	29.4	5.4	46.8
30	102.1	2.2	60.0	5.4	45.3
48	222.4	2.2	92.5	5.4	133.1

tion. In this series negative developing tests were obtained on both sera and cells of five stillborn infants clinically not erythroblastosis. In addition there was one hydropic stillborn from which neither serum nor erythrocytes could be obtained (Case 8). Clinical icterus was found in four infants negative to the developing test, each of which had the same Rh type as the mother. These were considered to be examples of physiologic jaundice.

In Table II are presented the results of the study of hemolytic action of Rh antibodies in vitro. It should be noted that significant results such as are shown here could not be obtained when complement and Rh antiserum were added to a 5 per cent suspension of the same Rh-positive erythrocytes in normal saline.

DISCUSSION

The sequence of events in the pathogenesis of erythroblastosis as outlined in this paper are now generally accepted. However, many factors concerning the maternal antibody response, the concentration of these antibodies in the fetal circulation, their adsorption on fetal erythrocytes, and the mode of destruction of these corpuscles, had not been entirely clear. For example, the lack of correlation between the severity of the hemolytic reaction and the maternal antibody titer and even the lack of demonstrable agglutinins had been noted by several authors.[22-25] It is believed that evidence is presented in this paper which sheds new light on some of these obscure phases of the erythroblastosis problem.

The successful removal of Rh antibodies from cord erythrocytes by elution in three cases of erythroblastosis is significant chiefly as a demonstration of the actual sensitization of the infant's cells preliminary to their destruction and as an opportunity to prove the specificity of this reaction by further readsorbing the eluted antibodies on known Rh-positive red cells. This readsorption may be indicated by agglutination or may require the blocking or developing tests, according to the type of Rh antibody involved (see Table I). This proof that the eluted material consisted of specific Rh antibodies of the same type found in the mother's serum indicates their origin in the mother as well as their transport across the placenta from the maternal circulation into that of the fetus. The specific nature of the union of the antibody to the Rh antigen of the erythrocyte is strongly suggestive of the destructive role this antibody must play in sensitizing the red cells for their hemolysis in vivo so characteristic of erythroblastosis. However, the actual hemolytic action of the Rh antibody in vitro had not been clearly demonstrated heretofore, although Diamond and Abelson[19] had noted hemolysis in doing their slide test with a heavy suspension of red cells.

In Table II the actual hemolysis of Rh-positive erythrocytes in vitro by the Rh antibody is shown by the significantly greater liberation of hemoglobin as compared to controls. Further studies on the hemolytic activity of Rh antibodies in vitro already completed will be reported elsewhere. However, these experiments seem to afford evidence that these antibodies can act as hemolysins as well as agglutinins in vitro. In comparing the relatively weak

hemolysis seen in vitro with the severe hemolysis common in vivo (erythroblastosis), it should be noted that in the latter cases red cell destruction may be greatly enhanced by the action of the spleen in its role of phagocytosis of slightly damaged corpuscles. Finally, the titer of 1:2,000 effective in the test tube is sometimes exceeded in mother's serum, particularly if the developing test is used to demonstrate the titer of anti-Rh immune globulin.

While the elution technique, in conjunction with the demonstration of hemolysis in vitro, presents additional evidence for the sequence of events in the pathogenesis of erythroblastosis, it seems to offer little as a test to identify antibodies in the infant's blood. Only in those cases that showed antibodies in the serum of the mother were the elution tests on fetal cells positive as described previously by the present authors[11] and by Carter and Loughrey.[13] In the series of cases herein described it was further demonstrated that positive elution tests to demonstrate antibody adsorbed on fetal cells were found only in those cases in which the cord serum as well as the maternal serum contained demonstrable Rh antibodies.

The whole problem of demonstration of antibodies in mother and child as well as attempts to correlate antibody titers with severity of erythroblastosis must now be re-examined in the light of new information obtained by the use of antihuman globulin serum as a developing test for Rh antibodies. Because the test yields observable agglutination it seems to offer some advantage over the blocking test in the clear-cut nature of the results and in its sensitivity. It was further suggested that the test might be used to detect sensitized red cells in the infant when Rh antibody could not otherwise be shown. Since adopting this method as a routine developing test in suspected Rh isoimmunizations, we have been able to confirm its advantages in respect to its sensitivity for incomplete antibodies and to prove its usefulness in demonstrating Rh antibody in the serum of mother and infant when other tests failed.

Of even greater interest is the fact that the developing test not only detects incomplete or blocking antibodies but also detects specific Rh antibodies not demonstrable with agglutinating or blocking tests. The developing test also shows agglutination when agglutinating Rh antibodies are present, but the clumping reaction is much stronger than that of the agglutinating antibody alone. In our cases it may be noted from Table I that the developing titer equalled, or far exceeded, the agglutination or blocking titers. In Cases 5 to 8 there are instances of developing titers in mother or baby much higher than agglutinating or blocking titers and even examples of positive tests when the other two tests were negative. This evident difference between the three tests indicates that the basic character of Rh antibodies must be different. In the case of the agglutinin which saturates the hapten and produces the visible reaction of clumping, the classical antibody is seen. In the case of the blocking reaction an antibody is present which is capable of specific hapten saturation without the production of clumping. However, in the case of the immune globulin demonstrable by the developing serum, the antibody apparently is capable of specific adsorption without saturation of the available haptens and without the

production of agglutination. That this latter reaction is specific can be proved by the fact that the adsorption of this immune globulin occurs only with the Rh antigen and not with the O, A, B, M, and N antigens. The unique feature of this developing reaction in not saturating the available haptens is further demonstrated by the fact that after Rh-positive cells are subjected to the immune globulin no interference is found with the agglutinability of these cells with known Rh-typing serum. For the sake of brevity and in line with the designation of agglutinating and blocking Rh antibodies, we have designated this third type as developing antibodies. It seems possible that these developing antibodies may represent a third order in respect to valence as compared to blocking and agglutinating antibodies, arising as incomplete or fractional units of the other two forms.

This developing antibody is of great clinical importance, since it has been observed by many investigators that clinical erythroblastosis may occur in the Rh-positive child of an Rh-negative mother in whom no antibodies were evident. Likewise, it has been noted that the observed titers of maternal antibodies have shown little or no correlation with the severity of the disease in the infant. Levine[23] wisely attributed this inconsistency to the limitations in the sensitivity of the techniques then available. The demonstration of the blocking effect by Race[17] and Wiener[18] gave the first evidence that we were dealing with immune substances previously hidden to the ordinary agglutinating test. When the developing test was applied to our cases (Table I), further evidence of hidden antibody titers became apparent. In Case 8, for example, the agglutinating titer of 1:2 and blocking titer of 1:32 certainly did not suggest the severe form of erythroblastosis represented by the macerated hydropic fetus. However, the developing titer of maternal antibodies of 1:2048 correlates well with the severity of the injury to the infant. In Cases 6 and 7 relatively mild clinical courses could be expected from the moderate developing titers. From the older Cases 1 to 5 conclusions must be drawn with caution because it may be assumed that considerable loss of titer may have occurred during the time the serum was preserved in the frozen state, although even here the developing titer was equal to, or greater than, the original agglutinating titer. The correlation of titer and severity of erythroblastosis must not be overstressed because the individual resistance of each baby involving questions of compensatory production of red cells, liver function,[27] and other factors is to be considered.

The application of the developing test to the erythrocytes of the newborn shows promise of giving us a specific test for erythroblastosis. The brief evidence to date suggests that this test may satisfy the requirements for sensitivity and specificity to fill this role. Since adoption as a routine test sensitized fetal red cells have been found in three cases, two with clinical hemolytic disease and one in subclinical form but with suggestive maternal history (Table I). In all four cases mothers were Rh negative and infants Rh positive. It should be noted that the developing test as applied to washed infant's cells is not necessarily specific for Rh antibodies but presumably would detect any specifically adsorbed antibody, for example, anti-Hr on Rh-negative cells or anti-A on A erythrocytes.

The developing test would also help avoid errors in instances such as Case 3 where blocking antibody adsorbed in vivo was sufficient to make the infant appear Rh negative. In such a case, of course, the developing test would be positive. Case 6 not only illustrates the diagnostic value of the developing test but also shows evidence of continuing adsorption of antibodies through the mother's milk. In this instance a strictly laboratory diagnosis of erythroblastosis was made on the basis of a 4 plus reaction of washed infant's cells to the antihuman globulin serum. The deterioration in the child's condition after discharge from the hospital and the continued strong developing test on the baby's cells can be attributed to the breast feeding. After stopping breast feeding the adsorbed antibodies were present nine days later, but seventeen days thereafter no sensitized infant erythrocytes could be found.

The specificity of the developing test is supported by the negative reactions obtained in stillborn infants and cases of jaundice of the newborn in which no evidence of erythroblastosis could be found. In most of these the Rh type was the same as the mother. If this specificity can be completely established in routine use, this test appears to be of the utmost practical value for diagnosis, prognosis, and treatment.

Analysis of the reported cases of hemolytic disease of the newborn brings out two therapeutic considerations. First, the use of early and adequate transfusion of Rh-negative blood, utilizing the cord route in severe cases, is urgently recommended. In Cases 3 and 9, with symptoms of kernicterus, transfusion was delayed three and two days, respectively. However, in Case 1, with a previous child suffering from kernicterus, a high maternal antibody titer, and strongly positive fetal eluate, the infant recovered completely. In this case a transfusion of 100 c.c. was given in the first hour after birth. Second, the infant should not be put on the breast if any Rh antibodies can be demonstrated, even if no clinical erythroblastosis is apparent. The developing test on infant erythrocytes probably will be sufficiently sensitive to determine this question for Rh-positive babies of Rh-negative mothers.

In addition to the use of developing serum in erythroblastosis, many other applications have been made in this laboratory. The results of the use of this method to bring out hidden titers in heterophile antibody tests and the Huddleson test for brucellosis will be reported elsewhere.

In transfusion reactions the developing serum has been useful in determinating whether a specific isoimmunization has occurred either to Rh or other unidentified antigens such as those reported by Race and associates.[26]

SUMMARY AND CONCLUSION

1. Additional evidence is presented which tends to establish certain steps in the pathogenesis of erythroblastosis.

2. Three varieties of Rh antibodies, namely, agglutinating blocking, and developing, found in the newborn as well as the mother are described.

3. Evidence is presented to show that the Rh antibody acts as a hemolysin in vitro.

4. Analysis of ten cases of erythroblastosis shows relatively good correlation between severity of the disease and antibody titer as demonstrated by the developing test.

5. Results of therapy in these cases suggests the advisability of immediate and adequate transfusion of Rh-negative blood in hemolytic disease of the newborn.

6. Breast feeding is contraindicated when Rh antibodies can be demonstrated in the maternal or fetal serum or adsorbed on fetal erythrocytes.

7. Evidence is presented indicating a high degree of sensitivity and specificity for the developing test as used herein to demonstrate isoantibodies against human erythrocytes.

8. The developing test as applied to the demonstration of adsorbed antibodies on fetal erythrocytes is suggested as a diagnostic test for erythroblastosis.

REFERENCES

1. Levine, P., and Stetson, R. E.: An Unusual Case of Intragroup Agglutination, J. A. M. A. **113**: 126-237, 1939.
2. Landsteiner, K., and Wiener, A. S.: Studies on an Agglutinogen (Rh) in Human Blood Reacting With Anti-Rhesus Sera and With Human Isoantibodies, J. Exper. Med. **74**: 309-320, 1941.
3. Levine, P., and Katzin, E. M.: Isoimmunization in Pregnancy and the Varieties of Isoagglutinins Observed, Proc. Soc. Exper. Biol. & Med. **45**: 343-346, 1940.
4. Levine, P., Katzin, E. M., and Burnham, L.: Atypical Warm Isoagglutinins, Proc. Soc. Exper. Biol. & Med. **45**: 346-348, 1940.
5. Levine, P.: Erythroblastosis Fetalis and Other Manifestations of Isoimmunization. West. J. Surg. **50**: 468-475, 1942.
6. Wiener, A. S., and Landsteiner, K.: Heredity of Variants of the Rh Type, Proc. Soc. Exper. Biol. & Med. **53**: 167-170, 1943.
7. Wiener, A. S., and Sonn, E. B.: Additional Variants of the Rh Type Demonstrable With a Special Human Anti-Rh Serum, J. Immunol. **47**: 461-465, 1943.
8. Witebsky, E., and Heide, A.: Further Investigations on Presence of Rh Antibodies in Breast Milk, Proc. Soc. Exper. Biol. & Med. **52**: 280-281, 1943.
9. Forbus, W. D.: Reaction to Injury. Pathology for Students of Disease, Baltimore, 1943, Williams & Wilkins Company, p. 42.
10. Levine, P.: Mechanism of the Isoimmunization by the Rh Factor of Red Blood Cells, Arch. Path. **37**: 82-90, 1944.
11. Haberman, S., and Hill, J. M.: The Clinical Significance of the Rh Factor. II. Its Importance in Erythroblastosis Fetalis, Texas State J. Med. **40**: 182-187, 1944.
12. Landsteiner, K., and Miller, C. P.: Serological Studies on the Blood of the Primates. II. The Blood Groups in Anthropoid Apes, J. Exper. Med. **42**: 853, 1925.
13. Carter, B. B., and Loughrey, J.: A Method of Demonstrating Anti-Rh Agglutinins in Cases of Erythroblastosis Fetalis, Am. J. Clin. Path. **15**: 575-576, 1945.
14. Coombs, R. R. A., Mourant, A. E., and Race, R. R.: Detection of Weak and "Incomplete" Rh Agglutinins: A New Test, Lancet **249**: 15-16, 1945.
15. Coombs, R. R. A., Mourant, A. E., and Race, R. R.: A New Test for the Detection of Weak and "Incomplete" Rh Agglutinins, Brit. J. Exper. Path. **26**: 255-266, 1945.
16. Coombs, R. R. A., Mourant, A. E., and Race, R. R.: In-vivo Isosensitisation of Red Cells in Babies With Hemolytic Disease, Lancet **250**: 264-266, 1946.
17. Race, R. R.: The Incomplete Antibody in Human Serum, Nature, London **153**: 771-772, 1944.
18. Wiener, A. S.: A New Test (Blocking Test) for Rh Sensitization, Proc. Soc. Exper. Biol. & Med. **56**: 173-176, 1944.
19. Diamond, L. K., and Abelson, N. M.: The Demonstration of Anti-Rh Agglutinins–An Accurate and Rapid Slide Test, J. Lab. & Clin. Med. **30**: 204, 1945.
20. Wiener, A. S.: Conglutination Test for Rh Sensitization, J. Lab. & Clin. Med. **30**: 662-667, 1945.
21. Bing, F. C., and Baker, R. W.: The Determination of Hemoglobin in Minute Amounts of Blood by Wu's Method, J. Biol. Chem. **92**: 589-600, 1931.

22. Wiener, A. S.: Hemolytic Reactions Following Transfusions of Blood of the Homologous Group, Arch. Path. **32**: 227-250, 1941.
23. Levine, P.: The Pathogenesis of Fetal Erythroblastosis, N. Y. State J. Med. **42**: 1928-1935, 1942.
24. Schwartz, H. A., and Levine, P.: Studies on the Rh Factor, Am. J. Obst. & Gynec. **46**: 827-835, 1943.
25. Gallagher, F. W., Davis, P. G., and Jones, L. R.: The Rh Factor in Relation to Jaundice of the Newborn Infant (Erythroblastosis Fetalis), J. Pediat. **22**: 171-174, 1943.
26. Race, R. R., Taylor, G. L., Cappell, D. F., and McFarlane, M. H.: Recognition of a Further Common Rh Genotype in Man, Nature, London **153**: 52, 1944.
27. Fashena, G. J.: The Mechanism of Hyperbilirubinemia of the Newborn. Read before The Society for Experimental Biology and Medicine, Southwestern Section, Oklahoma City, Okla., December, 1945.
28. Harville, C. H.: Cord Transfusion in the Management of a Prematurely Delivered Infant With Erythroblastosis, J. A. M. A. **129**: 801-802, 1945.

BLOOD TRANSFUSION STUDIES

I. The Differentiation of Hemolytic and Nonhemolytic Transfusion Reactions

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IN THE past the classification of transfusion reactions has depended mainly on the degree of fever and the severity of the clinical manifestations, but there has not been any general acceptance of a classification which differentiates reactions associated with hemolysis from those without hemolysis. The importance of examining the plasma for hemoglobinemia after every febrile transfusion reaction has been emphasized by various authors,[1-7] and the recognition of its occurrence and fundamental importance has been well known for years. Nevertheless, in many reports of fatal transfusion reactions information regarding hemoglobinuria is lacking, and in most reports no mention is made of hemoglobinemia.

The incidence of reactions of all kinds varies with different reports but usually ranges from 3 to 5 per cent. Erf and Jones[8] report an incidence of 3.2 per cent in a series of 2,889 transfusions. In this series only two transfusions resulting in nonfatal reactions were found to be due to incompatible blood, and urticaria developed in 0.3 per cent of the patients. In 1942 Levine[9] reported the incidence of reactions as 4.2 per cent of 522 consecutive blood transfusions and 2.0 per cent of 149 consecutive plasma infusions. Of these none were hemolytic. Wiener and co-workers[3] described two cases of incompatibility in 3,000 transfusions. Carlson[1] reported that 6.0 per cent of 3,388 transfusions of banked blood resulted in reactions. Of these eleven cases, or 0.32 per cent, were serious. These included three hemolytic reactions (one death), three cases of jaundice without other evidence of hemolytic reaction, two anaphylactic reactions, and three cases in which cardiovascular embarrassment was prominent. In a series of 43,284 transfusions collected from the literature, the incidence of hemolytic reactions was 1.8 per 1,000 transfusions and the mortality 1.4 per 1,000 transfusions.[10]

Wiener and Shaeffer[2] state that blood older than ten days caused chills and fever quite regularly. Icterus could not be detected in patients receiving blood less than eight days old, while in patients receiving older blood it occurred regularly, at times together with hemoglobinemia and hemoglobinuria. Most investigators agreed that unless the blood is older than nine days the age of blood does not influence the incidence of febrile reactions. DeGowin and associates[11] have demonstrated that the potassium content of the plasma of stored blood

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does not cause any untoward symptoms or signs. They have warned against the heating of donor's blood because of the occurrence of hemolytic reactions attributable to overheating of blood. No febrile reactions could be attributed to administration of blood at room temperature.

Hemolytic reactions are most often due to group incompatibility or to the occurrence of isoagglutinins, such as anti-Rh agglutinins, and occasionally to overheated blood. Hesse[12] stresses the use of Group O blood which has not been cross-matched as a cause of fatal reactions. Many of these reactions may have been due to the Rh factor, just as were many severe hemolytic intragroup reactions which were unexplained before discovery of the Rh factor. Aubert and co-workers[13] demonstrated that 40 per cent of Group O donors have anti-A titers of over 1:512; they noted that transfusions of Group O blood of such high anti-A titer, or higher, rarely produced an elevation of the red blood cell levels of Group A recipients. Severe or fatal hemolytic reactions did not occur. The use of low titer anti-A Group O plasma or blood is satisfactory. There have been many reports of the safe use of universal donors. In France the Transfusion Sanguine d'Urgence of Paris[14] had reported no fatalities due to hemolytic reactions up to 1939; Group O donors formed almost the sole source of blood for the service. Hardin[15] compared two series of transfusions in the Army Medical Service. There was no difference in the reaction rates between the 7,299 transfusions of group specific blood and the 9,392 transfusions of universal donor blood.

Transfusion reactions generally fall into the following groups: (1) simple febrile reactions due to pyrogenic substances in the solutions, (2) allergic reactions, both anaphylactic and urticarial, (3) circulatory reactions from too rapid administration particularly to seriously anemic and debilitated patients, and (4) febrile reactions accompanied by intravascular hemolysis. DeGowin[16] has also recognized a group with jaundice but without intravascular hemolysis. Separation of hemolytic reactions from the other varieties, and especially the simple febrile reactions, is often impossible on the basis of clinical symptoms and signs. The fever and chills may be equally severe, and the patient may appear just as ill with either form. Hesse[17] has stressed the importance of back pain and pain in the legs as evidence of spasm of the renal vessels immediately after a reaction to incompatible blood. It is true that many of the patients with transfusion reactions followed by renal insufficiency have varying degrees of backache and general body aches, but there are enough patients with no characteristic symptoms to make a differentiation difficult or impossible by symptomatology alone. Furthermore, aching pains may occur even after a pyrogenic reaction to plain crystalloid solutions administered intravenously. When blood is administered during anesthesia, fever alone may occur, the subjective reaction being entirely absent, and yet the patient may have a severe hemolytic reaction. When anuria occurs after an operation accompanied by transfusions, one should consider a hemolytic reaction as a likely cause and look for hemoglobinemia immediately in order to establish the diagnosis.

METHOD OF STUDY

In order to ascertain the significance of hemoglobinemia in reactions to transfusions, it was necessary to determine what would occur after ordinary transfusions. Therefore, a study was made of a series of fifty-eight transfusions given to patients with diseases associated with anemia. Blood specimens were obtained just before the transfusion was to be given and within the hour after its completion. The Malloy and Evelyn method[18] was used to determine serum bilirubin concentrations in forty-two patients before the blood was given at five hours and again from eighteen to twenty-four hours afterward. The blood for plasma was collected with a clean dry syringe and needle; 4.5 c.c. of blood were allowed to run by gravity into a graduated centrifuge tube containing 0.5 c.c. of 3 per cent sodium citrate solution. The tube was inverted gently only once and the cork removed. The blood was centrifuged immediately and the plasma volume recorded. The plasma was then separated from the cells using a medicine dropper.

The technique of hemoglobin determinations[19] is briefly as follows: The reagents are chemically pure pyridine, dilute ammonium hydroxide (1 per cent), from 0.2 to 0.4 per cent sodium hydrosulfite solution freshly made with dilute ammonia, and 3 per cent hydrogen peroxide. In an Evelyn colorimeter tube 1 c.c. (or less) of plasma is diluted to 7.5 c.c. with 1 per cent ammonium hydroxide, after which 0.5 c.c. of pyridine and 2.0 c.c. of fresh sodium hydrosulfite solution are added. A "blank" tube is set up simultaneously with all these ingredients, except that there is 1.0 c.c. less of ammonia to allow for 1.0 c.c. of 3 per cent hydrogen peroxide. Five minutes are allowed for decolorization of the contents of the "blank" tube. Using a 550 mμ filter, one determines the galvanometer deflection of the Evelyn colorimeter in the usual manner. The concentration is then determined as follows (appropriate correction has to be made if less than 1.0 c.c. of plasma is used):

$$\text{Concentration} = \frac{1000 \times \text{L}}{\text{K}} = \text{mg. hemoglobin per 100 c.c.}$$

L = (2 – logarithm of the galvanometer reading)
K = 1.843

The concentration is corrected for dilution by multiplying the value obtained by the factor: $\frac{\text{Volume of supernatant fluid}}{\text{Volume of supernatant fluid} - 0.5 \text{ c.c.}}$. Normal values with this method range from a trace to 6.0 mg. per 100 c.c. of plasma. Normal values obtained by the benzidine method of Bing and Baker[20] range from a trace to 5.0 mg. per 100 c.c. of plasma.

In four of fifty-eight patients with anemia the plasma hemoglobin level before transfusion was higher than 6.0 mg. per 100 cubic centimeters. Determinations on the plasma of each of these patients were repeated within an hour afterward and before the time of another transfusion and were found to be less than 6.0 mg. per 100 c.c., so it is safe to conclude that slight artificial hemolysis had occurred at the time of the collection of the first blood specimen

TABLE I. HEMOGLOBIN LEVELS BEFORE TRANSFUSION

MG. HB. PER 100 C.C. PLASMA	NUMBER	PER CENT
0 to 3	48	83.0
3.1 to 6	6	10.2
6.1 to 9	2	3.4
9.1 to 12	2	3.4
Total	58	100.0

(see Table I for the distribution of values for plasma hemoglobin before transfusion). It is important to note that hemoglobin is not visible in plasma until the level is higher than 20 to 25 mg. per 100 c.c.; therefore, hemoglobin is not visible in properly obtained normal plasma or serum but is easily visible when any serious intravascular hemolysis has occurred recently.

In Table II is shown the range of hemoglobin concentrations in specimens of plasma obtained within the first hour after transfusion. Old blood is used to designate bank blood older than three days. There are a few values above 6.0 mg. but none higher than 12.0 mg. per 100 cubic centimeters. There was an average increase over the pretransfusion values of about 1.0 mg. per 100 cubic centimeters. This can be accounted for in part by the fact that a small amount of hemoglobin was found in the transfused plasma. It is safe to say that no intravascular hemolysis occurred in any case.

Transfusions With Febrile Nonhemolytic Reactions.—Nineteen febrile reactions to transfusions were studied, which proved to be nonhemolytic (see Table II). Nine patients had a temperature elevation to 103° F. or higher, and all had chills and some fever. Specimens were obtained within an hour in each instance, and in some instances later specimens were also obtained. The distribution of values is practically the same as those obtained after uncomplicated transfusions. It was gratifying to know that no hemolysis had occurred, even though some of the reactions were very severe from the standpoint of clinical symptoms.

TABLE II. PLASMA HEMOGLOBIN LEVELS AFTER EIGHTY-TWO TRANSFUSIONS

MG. HB. PER 100 C.C. PLASMA	GROUP WITH NO REACTIONS						GROUP WITH FEBRILE REACTIONS	
	OLD BLOOD		FRESH BLOOD		TOTAL			
	NO.	PER CENT	NO.	PER CENT	NO.	PER CENT	NO.	PER CENT
0 to 3	15	62.5	24	61.5	39	62.0	7	36.8
3.1 to 6	4	16.7	9	23.0	13	20.6	9	47.4
6.1 to 9	3	12.5	5	12.8	8	12.7	2	10.5
9.1 to 12	2	8.3	1	2.7	3	4.7	1	5.3
Total	24	100.0	39	100.0	63	100.0	19	100.0

Transfusions With Hemolytic Reactions.—In Table III are summarized some of the pertinent data in five cases of hemolytic reactions to transfusion studied during the years 1942 to 1944. Patients L. H. and M. K. did not develop hemoglobinuria. Patient L. H. was given 200 c.c. of blood before she had a severe chill, pain in the back, and a fever of 104° Fahrenheit. Patient M. K. was given 500 c.c. of blood before she had a severe chill and a fever of 103.6° Fahrenheit. It is possible that Patient M. K. had a higher level than 124 mg. per 100 c.c., because three hours had elapsed after the reaction. At any rate the threshold

TABLE III. HEMOLYTIC REACTIONS TO TRANSFUSION

PATIENT	TIME AFTER TRANSFUSION (HR.)	PLASMA HEMOGLOBIN (MG./100 C.C.)	METHEMALBUMIN	OLIGURIA	HEMOGLOBINURIA	BILIRUBIN (MG./100 C.C.)
L. H.	½	36				
	2	38				
	12			0	0	2.5
M. K.	3	124				
	5	75				3.3
	18			0	0	2.7
J. N.	24	54			+	
	30	43	0	0	0	
	40	12			0	4.6
M. B.	42	38	0	0	+	
	48	33			0	6.1
	67	16			0	
	96					.47
J. L.	20	254	0	+	+	
	44	160	+	+	+	Jaundice

for hemoglobin was not exceeded in either instance, and the true nature of the reaction would have been unrecognized if hemoglobinemia had not been found. Neither patient was jaundiced beforehand, but, as indicated in Table III, transient elevation of bilirubin values afterward substantiated the occurrence of a hemolytic episode. Visible jaundice was very slight or questionable.

The last three patients received transfusions during major operations, so that they did not manifest the usual clinical evidence of reaction.

Patient J. N. was given 500 c.c. of Group O blood, 540 c.c. of Group O plasma and 250 c.c. of Group A plasma during an operation for the ligation of a patent ductus arteriosus. It may be noted that the first plasma specimen was obtained twenty-four hours afterward and that hemoglobinuria was present up to this time but absent thereafter.

Patient M. B. was given 2,000 c.c. of blood during a lobectomy for bronchial adenoma. Hemoglobinuria continued until the forty-second hour but was absent thereafter. In patients J. N. and M. B., hemoglobinuria was responsible for the recognition of hemolysis but this was a long time after the transfusions in both instances. The serum bilirubin concentrations were increased in the first four patients after the transfusion reactions but quickly returned to normal. The bilirubin was indirect reacting in each instance so only the total bilirubin is recorded. Recovery was uneventful in each of the first four instances. No signs of renal damage occurred. Neither Patient J. N. nor Patient M. B. had methemalbumin[21] in the plasma on ordinary spectroscopic examination, but Schumm's test for hemochromogen was not carried out.

Patient J. L. was given multiple transfusions during a craniotomy. Oliguria and hemoglobinuria were noted after operation. Severe oliguria continued until the patient's death seventy-six hours later; only 25 to 35 c.c. of urine were obtained by catheter during each twenty-four hour period. The blood urea nitrogen was 103 mg. per cent just before death. Jaundice was present, but the quantity of bilirubin was not determined. Methemalbumin[21] with an absorption band at 623 to 624 mμ was recognized in the plasma specimens at forty-

four hours and sixty-eight hours after the first transfusion but was not present at twenty-four hours. The addition of sodium cyanide to the plasma did not abolish the characteristic band nor change its intensity visibly, so that it could not have been due to methemoglobin. Unfortunately, permission for autopsy could not be obtained in this case.

It is not possible to estimate the initial plasma hemoglobin levels in these last three instances; however, since hemoglobin is cleared very rapidly from the blood stream, it may be assumed that the initial levels must have been much higher in each case. Obviously Patient J. L. suffered the most severe reaction as evidenced by the plasma hemoglobin levels and the fatal renal injury with urinary suppression. Gilligan and associates[22] injected 16 Gm. of hemoglobin into an adult, and the plasma was cleared of hemoglobin in twelve hours. If all the cells in 500 c.c. of blood were hemolyzed, from 60 to 75 Gm. of hemoglobin would be liberated giving plasma levels of 2,000 to 2,500 mg. per 100 c.c. (assuming a plasma volume of 3 liters).

DISCUSSION

The absence of hemoglobinemia following transfusions which do not result in any kind of reaction has been demonstrated. The absence of hemoglobinemia after certain febrile transfusion reactions is important evidence in separating such reactions from hemolytic reactions. Two patients with hemolytic reactions and early, transient hemoglobinemia and without hemoglobinuria substantiate the contention that hemoglobinemia is a fundamental and pathognomonic feature of intravascular hemolysis. One patient developed anuria and died as a result of uremia. This patient had the highest plasma hemoglobin levels observed in any of the five patients with hemolytic reactions; this observation lends support to the thesis that the plasma hemoglobin level is an important factor in the development of renal damage. In each of the last three cases cited observations were made a long time after the transfusions, so one cannot compare the findings with any observations except those made at the same time interval, since hemoglobin disappears from the blood stream at a rapid rate.

Although infrequent, hemolytic transfusion reactions are the chief hazard of blood transfusions. It is important to recognize the occurrence of hemoglobinemia whenever intravascular hemolysis has occurred and to obtain and examine the serum or plasma immediately after any reaction to transfusion. To insure against artificial hemolysis, one can use isotonic sodium citrate as described under Method of Study. It would be very instructive to make serial quantitative plasma hemoglobin determinations whenever intravascular hemolysis has been sufficiently great to color the plasma. The quantity of hemoglobin excreted in the urine should be determined at appropriate intervals depending on the individual case.

Whenever a febrile nonhemolytic reaction to transfusion has occurred, every effort should be made to determine its cause. The contents of the transfusion set should be examined for bacterial contamination. The tests for compatibility of donor and recipient should be repeated carefully. The patient should be observed for the development of signs of shock, and the fluid intake and output should be recorded carefully.

CONCLUSIONS

Hemoglobinemia does not result from transfusion of fresh compatible blood. The importance of distinguishing hemolytic from nonhemolytic transfusion reactions has been emphasized. Hemolytic reactions are characterized by hemoglobinemia, whereas simple febrile reactions are not. There is a need for more quantitative observations in patients with hemolytic transfusion reactions made immediately after the reaction and followed serially until hemoglobinemia disappears. All febrile reactions should be studied early and every effort made to determine the etiology of the reaction.

REFERENCES

1. Carlson, R. I.: Reactions to Transfusions of Banked Blood, Two Years' Experience of the New Haven Hospital Blood Bank, Yale J. Biol. & Med. **16:** 167, 1943.
2. Wiener, A. S., and Schaeffer, G.: Limitations in the Use of Preserved Blood for Transfusions. A Study of the Fate of Transfused Erythrocytes in the Recipient's Circulation, M. Clin. North America **24:** 705, 1940.
3. Wiener, A. S., Oremland, B. H., Hyman, M. A., and Sanwick, A. A.: Transfusion Reactions. Experience With More Than 3000 Blood Transfusions, Am. J. Clin. Path. **11:** 102, 1941.
4. Wiener, A. S., Silverman, I. J., and Aronson, W.: Hemolytic Transfusion Reactions. II. Prevention With Special Reference to a New Biological Test, Am. J. Clin. Path. **12:** 241, 1942.
5. Mollison, P. L.: Die Nebenerscheinungen bei Bluttransfusionen, Bull. War Med. **4:** 341, 1945 (abstract).
6. Strumia, M. M.: The Fate of Transfused Refrigerated Blood and the Problem of Blood Banks, S. Clin. North America **22:** 1693, 1942.
7. Mollison, P. L.: The Investigation of Haemolytic Transfusion Reactions, Brit. M. J. **1:** 529, 559, 1943.
8. Erf, L. A., and Jones, H. W.: Experiences With the Blood Transfusion Unit in a 700-Bed Hospital: An Annual Survey of Over 3,500 Administrations of Blood and Plasma (Dried), Ann. Int. Med. **19:** 1, 1943.
9. Levine, M.: Blood Bank Problems, Univ. Minn. Staff Meet. Bull. **13:** 154, 1942.
10. Kilduffe, R. A., and DeBakey, M.: The Blood Bank and the Technique and Therapeutics of Transfusions, St. Louis, 1942, The C. V. Mosby Company, p. 481.
11. DeGowin, E. L., Hardin, R. C., and Harris, J. R.: Studies on Preserved Human Blood. III. Toxicity of Blood With High Plasma Potassium Transfused Into Human Beings, J. A. M. A. **114:** 858, 1940.
12. Hesse, E.: Ueber die Verwendung des sogennanten Universalspenders bei der Bluttransfusion, Deutsche Ztschr. f. Chir. **245:** 371, 1935.
13. Aubert, E. F., Boorman, K., Dodd, B. E., and Loutit, J. F.: The Universal Donor With High Titer Iso-agglutinins. The Effect of Anti-A Iso-agglutinins on the Recipients of Group A, Brit. M. J. **1:** 659, 1942.
14. Cited by Aubert and associates.[13]
15. Hardin, R. C.: Reactions From Universal Donor Blood, Proc. Central Soc. Clin. Research **18:** 19, 1945; abstracted from J. Lab. & Clin. Med. **31:** 472, 1946.
16. DeGowin, E. L.: Isoimmunity to the Rh Factor as a Cause of Blood Transfusion Reactions, J. Lab. & Clin. Med. **30:** 99, 1945.
17. Hesse, E.: Ueber das Wesen und Behandlung des Hamolytischen Schocks nach Bluttransfusion im Lichte experimenteller und Klinischer Forschung, Beitr. z. klin. Chir. **163:** 390, 1936.
18. Malloy, H. T., and Evelyn, K. A.: The Determination of Bilirubin With the Photoelectric Colorimeter, J. Biol. Chem. **119:** 481, 1937.
19. Flink, E. B., and Watson, C. J.: A Method for the Quantitative Determination of Hemoglobin and Related Heme Pigments in Feces, Urine and Blood Plasma, J. Biol. Chem. **146:** 171, 1942.
20. Bing, F. C., and Baker, R. W.: The Determination of Hemoglobin in Minute Amounts of Blood by Wu's Method, J. Biol. Chem. **92:** 589, 1931.
21. Fairley, N. H.: Methaemalbumin. I. Clinical Aspects, Quart. J. Med. **10:** 95, 1941. Fairley, N. H.: Methaemalbumin. II. Its Synthesis, Chemical Behavior, and Experimental Production in Man and Monkeys, Quart. J. Med. **10:** 115, 1941.
22. Gilligan, D. R., Altschule, M. D., and Katersky, E. M.: Studies of Hemoglobinemia and Hemoglobinuria Produced in Man by Intravenous Injection of Hemoglobin Solutions, J. Clin. Investigation **20:** 177, 1941.

BLOOD TRANSFUSION STUDIES

II. The Survival of Fresh and Stored Transfused Erythrocytes as Determined by Urobilinogen Excretion

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NUMEROUS studies have been devoted to the fate of the transfused erythrocyte, the effect of storage on its in vitro properties, and the effectiveness of various preservative solutions. The most important criterion for assessing the value of the transfused erythrocyte is its in vivo survival time. The fate of the transfused erythrocyte has been studied by three methods: the differential agglutination method of Ashby[1] and Wiener[2]; the use of radioactive iron which is incorporated in the hemoglobin molecule[3]; and the study of urobilinogen excretion following transfusion.[4]

In Ashby's agglutination method a Group A or Group B recipient is transfused with Group O blood. Periodically thereafter small samples of blood are taken. The recipient's cells are selectively agglutinated with Group O serum leaving the donor's cells free to be counted. Wiener's method[2] is similar in principle but makes use of the M and N subtypes of blood.

Recently Ross and Chapin[3] have utilized the radioactive isotope of iron for the identification of transfused blood cells. When fed or injected into persons with hypochromic anemia of simple iron deficiency, this isotope is incorporated into the hemoglobin of newly formed erythrocytes. Blood was drawn from these donors into flasks containing 2.5 per cent sodium citrate solution with a pH of 7.4 and stored for periods varying from one to fourteen days at 10° C. in a dark refrigerator. From 40 to 50 c.c. of this labeled blood were injected intravenously into healthy adults all of whom had normal hemoglobin levels. At varying intervals of time after the injection of blood, samples were withdrawn for blood volume determinations and for counts of radioactive cells by the use of the Geiger-Müller apparatus.

The study of urobilinogen excretion before and after transfusion was used in the present study and will be discussed in more detail later.

It is generally agreed by most investigators[1-3, 5, 6, 10, 12] that the average in vivo survival time of fresh transfused erythrocytes is more than thirty days and that some survive as long as 120 days. Fresh blood is composed of cells of varying ages, and their potential life is inversely proportional to their age, even under the most favorable circumstances. Data collected by Mollison and Young[6, 7] show that about 85 per cent of fresh transfused blood cells are still present at the end of two weeks, 70 per cent at the end of four weeks, and 45 per cent at the end of eight weeks.

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As the storage time of citrated blood increases the red cell survival time decreases. The data of various investigators on the survival time of stored citrated blood of varying ages are not in complete agreement and do not lend themselves very well for comparison. However, from the data available, it appears that citrated blood stored three days or less is equal to fresh blood in respect to survival. According to Mollison and Young,[7] citrated blood five to nine days of age has a fairly good survival; that is about 50 per cent of the cells were still viable at three weeks and 27 per cent at the end of two months. On the other hand, Belk and Barnes[5] state that blood stored seven days or more in sodium citrate solution survived no more than forty-eight hours. Citrated blood stored between four and six days occupies an intermediate position and probably should be considered only moderately effective in respect to survival time.

Ross and Chapin[3] studied the twenty-four hour survival of erythrocytes by means of radioactive iron and found that only 60 per cent of six-day-old cells and about 5 per cent of ten-day-old or older cells survived for twenty-four hours, that is, were found in the circulating blood.

As early as 1916 Rous and Turner[11] found that the addition of glucose to the citrated blood of experimental animals enhanced the keeping qualities and the in vivo survival of transfused blood. However, the large volume of citrate and glucose solution used by them made it impractical. Since then glucose-citrate solutions of smaller volume have been used with excellent results in regard to convenience and survival of the transfused erythrocytes.[9] When these mixtures are used the cell survival time depends somewhat upon the concentration of glucose and citrate, but the optimal range is quite wide. It has been found that variation of the concentration of glucose in the final mixture from 0.6 to 2.2 per cent makes little difference to subsequent survival. A citric acid-sodium citrate-glucose mixture can be autoclaved without caramelization of the glucose, but a sodium citrate-glucose mixture will be caramelized by autoclaving. Immediate storage of donors' blood at a temperature between 4 and 10° C. and avoidance of actual freezing are essential.

Dacie and Mollison[12] showed that blood taken from a patient with familial hemolytic anemia both before and after splenectomy disappeared rapidly when transfused into a normal recipient. On the other hand, normal blood transfused into six patients with familial hemolytic anemia survived normally in five, and in one patient cell survival was somewhat diminished.

PIGMENT STUDIES FOLLOWING FRESH AND OLD BLOOD TRANSFUSIONS

Strumia[13] and Wiener and Schaeffer[14] have noted hemoglobinemia following transfusion of citrated blood older than ten days. Transient bilirubinemia has also been observed following the use of blood stored for more than five days compared to that stored for shorter periods.[14] Wasserman and associates[4] in a similar study on three patients with hyporegenerative anemia found an increase in feces urobilinogen excretion roughly proportional to the time of storage of citrated blood. Fresh blood usually caused no, or only a very slight, increase

in urobilinogen excretion. In their last case[4] blood stored in glucose-citrate mixtures caused less rise in urobilinogen than when stored in citrate alone.

In order to study the relative efficiency of the blood in the University Hospital blood bank, the values of the serum bilirubin, plasma hemoglobin, and feces urobilinogen were observed before and after fresh and old blood transfusions. The donor blood was drawn into sterile flasks containing 50 c.c. of 2.5 per cent sodium citrate and stored at 4 to 10° Centigrade. Although not as accurate quantitatively as the actual counting of surviving donor cells, the urobilinogen excretion roughly indicates the rate of cellular destruction after transfusion. After the preliminary studies, fresh blood (stored less than twenty-four hours) was given over a period of one to one and one-half hours. Plasma hemoglobin levels and serum bilirubin were determined within an hour and again five hours after transfusion. Quantitative feces urobilinogen studies were carried out for the next eight days. After an interval of eight days or longer, the same studies were repeated following transfusion of old blood (stored from six to eight days). The amount of blood was 450 c.c., except in one case where two transfusions were given in succession instead of one. The urobilinogen was determined in each instance by the method of Watson,[15] the bilirubin by the method of Malloy and Evelyn,[16] and the plasma hemoglobin by the method of Flink and Watson.[17]

The first four patients had severe hyporegenerative or aplastic anemia. Patient H. had diabetes mellitus and chronic osteomyelitis, and Patient C. T. had chronic glomerulonephritis with uremia. None of the patients experienced untoward symptoms following these transfusions.

RESULTS

The plasma hemoglobin showed no appreciable change following any of the transfusions. No significant intravascular hemolysis took place following either fresh or old blood transfusions (Table I).

TABLE I

PATIENT	INITIAL HEMOGLOBIN (GM./100 C.C.)	MAXIMUM PLASMA HEMOGLOBIN (MG./100 C.C.)		INCREASE IN BILIRUBIN CONCENTRATION (MG./100 C.C.)	
		BEFORE	AFTER	FRESH BLOOD	OLD BLOOD
B. D.	5.84	7.0	1.4	.39	2.55
L. M.	2.2	1.5	3.4	.32*	2.8
C. A.	4.35	1.0	4.2	.0	1.68
O. H.	4.15	1.1	9.0	.35*	.51*
H.	8.6	2.7	6.8	.20	.84
C. T.	4.4	1.3	3.9	.18	.45

*Average of two transfusions on successive days.

In all cases studied there was a greater rise in the bilirubin concentration after the old blood than after fresh blood. The maximum bilirubin concentration was found usually at five hours after transfusion. This finding is in agreement with the observations of others.[13, 14]

TABLE II. UROBILINOGEN EXCRETION IN FECES (MG. PER DAY)

PATIENT	FRESH BLOOD TRANSFUSIONS			OLD BLOOD TRANSFUSIONS		
	BEFORE	AFTER	INCREASE	BEFORE	AFTER	INCREASE
B. D.	138	103	–	139	234	95
L. M.*	45	65†	20	131	268	137
C. A.	71	73	2	72	139	67
O. H.‡	157	98†	–	63	134†	72
H.	55	26	–	42	163	121
C. T.	47	128	81	88	145	57
Average	85	82		89	181	92

*Transfusions on two successive days, fresh blood.

†Result after two transfusions on successive days, the four-day period beginning with the first transfusion.

‡Transfusions on two successive days for both the fresh and old blood studies.

Feces urobilinogen excretion (Table II) was considerably increased after old blood but not after fresh blood in Patients B. D., L. M., C. A., and H. Patient O.H. excreted more urobilinogen after old blood than in the control period but excreted less than the control value obtained before the first transfusion. In Patient C. T. the increase in urobilinogen was slightly greater after fresh blood than after old blood. In five out of six patients studied it was apparent that an increased rate of destruction of blood must have taken place after the use of old blood compared with that following fresh blood.

CONCLUSIONS

It is evident that there is an increased extravascular destruction of red blood cells after transfusion of old blood, for there is a significant rise in the serum bilirubin and feces urobilinogen excretion after such a transfusion. Blood stored in sodium citrate for more than six days is less efficient than fresher blood in the therapy of anemias of any kind. This is supported by the pigment studies previously described and also by the studies of others, based on differential agglutination and the use of radioactive iron.

REFERENCES

1. Ashby, W.: The Determination of the Length of Life of Transfused Blood Corpuscles in Man, J. Exper. Med. **29**: 267, 1919.
2. Wiener, A. S.: Longevity of the Erythrocyte, J. A. M. A. **102**: 1779, 1934.
3. Ross, J. F., and Chapin, M. A.: Effect of Storage of Citrated Blood on the Survival of Transfused Erythrocytes, J. A. M. A. **123**: 827, 1943.
4. Wasserman, L., Volterra, M., and Rosenthal, N.: Quantitative Urobilinogen Excretion Following Transfusion of Fresh and Stored Blood, Am. J. M. Sc. **204**: 356, 1942.
5. Belk, W. P., and Barnes, B. C.: The Survival Time After Transfusion of Erythrocytes of Citrated Human Blood Stored at 4° to 6° Centigrade, Am. J. M. Sc. **201**: 838, 1941.
6. Mollison, P. L., and Young, I. M.: On the Survival of Transfused Erythrocytes of Stored Blood, Quart. J. Exper. Physiol. **30**: 313, 1941.
7. Mollison, P. L., and Young, I. M.: In Vivo Survival in the Human Subject of Transfused Erythrocyte After Storage in Various Preservative Solutions, Quart. J. Exper. Physiol. **31**: 359, 1942.
8. Loutit, J. F., and Mollison, P. L.: Advantages of a Disodium Citrate Glucose Mixture as a Blood Preservative, Brit. M. J. **2**: 744, 1943.
9. Loutit, J. F., Mollison, P. L., and Young, I. M.: Citric Acid-Sodium Citrate-Glucose Mixtures for Blood Storage, Quart. J. Exper. Physiol. **32**: 183, 1934.
10. Denstedt, O. F., Osborne, D. F., Stansfield, R., and Rochlin, I.: Survival of Preserved Erythrocytes After Transfusion, Canad. M. A. J. **48**: 477, 1943.

11. Rous, P., and Turner, J. R.: The Preservation of Living Red Cells in Vitro. II. Transfusion of Kept Cells, J. Exper. Med. **23**: 239, 1916.
12. Dacie, J. V., and Mollison, P. L.: Survival of Normal Erythrocytes After Transfusion to Patients With Familial Hemolytic Jaundice, Lancet **1**: 550, 1943.
13. Strumia, M. M.: The Fate of Transfused Refrigerated Blood and the Problem of Blood Banks, Surg. Clin. North America **22**: 1693, 1942.
14. Wiener, A. S., and Schaeffer, G.: Limitation in Use of Preserved Blood for Transfusions, Med. Clin. North America **24**: 65, 1940.
15. Watson, C. J.: An Improved Method for the Quantitative Estimation of Urobilinogen in Feces and Urine, Am. J. Clin. Path. **6**: 458, 1936.
16. Malloy, H. T., and Evelyn, K. A.: The Determination of Bilirubin With the Photoelectric Colorimeter, J. Biol. Chem. **119**: 481, 1937.
17. Flink, E. B., and Watson, C. J.: A Method for the Quantitative Determination of Hemoglobin and Related Heme Pigments in Feces, Urine and Blood Plasma, J. Biol. Chem. **146**: 171, 1942.

BLOOD AND BONE MARROW IN INFECTIOUS MONONUCLEOSIS*

A Review of the Literature and a Report of Twenty-Five Cases

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SINCE the original descriptions of infectious mononucleosis by Pfeiffer[1] (1889) and others before the turn of the century, an enormous literature has accumulated pertaining to the clinical aspects and laboratory characteristics of the disease. The introduction by Paul and Bunnell[2] (1932) of the serologic test whereby suspected cases of infectious mononucleosis could be diagnosed without question served to stimulate new interest in the disease.

Although numerous articles in the literature touch on the hematologic findings in infectious mononucleosis, none of them have added much to Downey's[3] classical morphologic description of the blood. His original colored plate with the description of the cells seen in this disease compared with those seen in cases of leucemia has never been surpassed.

Studies of the bone marrow in cases of infectious mononucleosis have been surprisingly few in number. It is only in the last ten years that any attempt has been made to correlate the blood findings with the cellular pattern in the bone marrow. It is of interest to note, in view of the fact that the disease may be mistaken for leucemia, that the first examinations of the bone marrow in this disease were made by Freeman[4] (1936) who suggested that infectious mononucleosis may really be an abortive, benign form of acute lymphatic leucemia. It is surprising that no one has challenged this investigator's conception of the malignant potentiality of the atypical lymphocytes seen in infectious mononucleosis. Read and Helwig[5] analyzed 300 cases of infectious mononucleosis and without examining the bone marrow arrived at the conclusion that the cause of the anemia, leucopenia, and thrombopenia in some of their cases was due to depression of the activity of the bone marrow resulting from an infectious granulomatous process involving the marrow.

Review of the Literature on the Bone Marrow in Infectious Mononucleosis

Freeman[4] was the first to describe the bone marrow in cases of infectious mononucelosis. He reported two cases, one in a 19-year-old student nurse with a leucocyte count of 10,400 per cubic millimeter and 76 per cent lymphocytes and a second case in a 55-year-old white woman with a white blood count of 23,600 per cubic millimeter and 91 per cent lymphocytes. The leucocyte count

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in the second case rapidly increased to 392,000 per cubic millimeter, and the woman died on the seventeenth day of illness. The heterophile antibody test was not performed in either of the two cases. The bone marrow of the sternum in both cases was filled with both immature and mature lymphocytes. Histologic sections of the bone marrow showed a lymphocytic infiltration. Freeman suggested that glandular fever may really be an abortive, benign form of acute lymphatic leucemia.

Young and Osgood[6] stated that in infectious mononucleosis the typical large lymphocytes were demonstrated to be present in the sternal marrow as well as in the blood. There is no mention as to the number of cases they studied.

Nordenson[7] examined the sternal marrow in thirteen cases of infectious mononucleosis and observed a toxic alteration of the myeloid cells with a moderate shift to the left of the granulocytic series. Erythropoiesis was normal. There were no morphologic indications of a lymphoid metaplasia in the bone marrow of the sternum.

Markoff[8] reported on the bone marrow findings in one case of infectious mononucleosis. He observed a lymphatic and plasma cellular reaction of the marrow with an increase in the number of metamyelocytes and a decrease in all the other granulopoietic elements. Erythropoiesis and megakaryopoiesis appeared normal. According to this investigator, in spite of the increased numbers of lymphoid cells in the sternal marrow in cases of infectious mononucleosis, the differential diagnosis from the bone marrow in lymphatic leucemia is relatively simple, and there is no reason for confusing the two diseases.

Rohr[9] who studied twelve cases of infectious mononucleosis emphasized that great care should be exercised in interpreting the cellular marrow pattern in this disease because of the variations in the marrow pattern which are directly related to the amount of admixture with peripheral blood. The bone marrow in his cases with the disease revealed a moderate amount of myeloid immaturity. He never observed a lymphoid infiltration of the sternal marrow in his cases of infectious mononucleosis.

Mallarmé[10] believes that sternal puncture aids in the diagnosis of infectious mononucleosis. The bone marrow in Mallarmé's case showed a myeloid reaction with an increase in the number of mononuclear cells, large lymphocytes, monocytes, and plasma cells. Granulopoiesis appeared normal.

Weil and Perlés[11] observed a hypoplastic bone marrow which consisted of myeloerythroblastic tissue and mononuclear cells similar to those seen in the peripheral blood and spleen.

Schulten,[12] Klima,[13] and Henning[14] have observed varying degrees of myeloid immaturity and confirmed Rohr's[9] observation of the lack of any evidence of a lymphoid infiltration of the bone marrow in cases of infectious mononucleosis.

Henning and Keilhack[15] observed a case of infectious mononucleosis with anemia, thrombocytopenia, and a leucocyte count of 30,000 per cubic millimeter with 80 per cent lymphocytes. The sternal aspiration was rich in cells of the granulocytic series with a few elements belonging to the mononuclear series.

Ustvedt[16] studied the blood and bone marrow in one case of infectious mononucleosis. This 29-year-old man had a leucocyte count that varied between 7,000 and 10,000 per cubic millimeter and the differential count showed between 60 and 89 per cent atypical lymphoid cells. The bone marrow was hypocellular and consisted of granulocytes in various stages of development, a few erythroid cells, and lymphoid cells. The uniform type of "blast" cells seen in the marrow in cases of acute leucemia were absent. Ustvedt observed groups of mononuclear cells of a lymphoid type in the bone marrow of his case of infectious mononucleosis which were quite different from those seen in the peripheral blood and concluded that the question of lymphoid metaplasia in the bone marrow could not be eliminated. He suggested sternal puncture as an aid in ruling out leucemia.

Fieschi[17] examined the bone marrow in a case of infectious mononucleosis and found a cellular marrow with a decrease in the number of mature neutrophiles and a few atypical granuloblasts similar to those seen in cases of acute infection. Lymphocytes were not observed, and plasma cells were few in number. The monocytoid cells seen in the marrow were morphologically different from the lymphoid cells found in the peripheral blood. The marrow mononuclear cells presented marked immaturity which suggested their origin from reticular cells or from large atypical hemocytoblastic elements. The bone marrow in no way simulated that seen in leucemia.

Scott[18] observed a normal cellular pattern in a case of infectious mononucleosis. He believes the method of sternal puncture has little value in diagnosing this condition, for even the findings of a normal marrow do not exclude lymphoid leucosis.

Vogel, Erf, and Rosenthal[19] examined the sternal marrow in six cases of infectious mononucleosis and found an increased number of lymphocytes. Many of the lymphocytes were of the lymphoid type. In one case Türk irritation cells were seen in the blood but were absent in the marrow, while in a second case Türk cells were present in both the blood and bone marrow. In a second article, Vogel and Bassen[20] reported the marrow findings in four cases of infectious mononucleosis. They found a normal number of lymphoid elements in the sternal marrow. These investigators believe that the absence of an increased number of lymphocytes in the bone marrow is of great value in ruling out the possibility of leucemia, especially when the heterophile antibody reaction is negative.

Morrison and Samwich[21] studied the marrow in eight cases of infectious mononucleosis and noted a left shift in the white blood series with increased red cell activity. There were many lymphocytes in the marrow, but they were never in the proportion as seen in lymphatic leucemia. The erythroid series revealed a defective maturation which the investigators ascribed to a lack of antianemia principle or to some disturbance in its utilization or absorption.

Mendell, Meranze, and Meranze[22] reported the bone marrow findings in eleven cases of infectious mononucleosis and observed a normal leucopoiesis and erythropoiesis. The total marrow cell count ranged from 20,000 to 170,000 per cubic millimeter, and the differential count showed 1 per cent myeloblasts, 2 per cent promyelocytes, 17 per cent myelocytes, 25 per cent metamyelocytes, 36 per

cent neutrophiles, 13.7 per cent lymphocytes (normal according to these investigators is 13.5 per cent), 3 per cent monocytes, 2 per cent eosinophiles, and 0.3 per cent reticulum cells. The erythroid series consisted of 1 megaloblast, 5 erythroblasts, and 12 normoblasts per 100 white cells. According to these authors the finding of a normal bone marrow in infectious mononucleosis, despite the large number of lymphoid cells in the peripheral blood, is sufficient to eliminate the possibility of leucemia.

Piney[23] from his studies of the bone marrow in infectious mononucleosis (number of cases studied is not stated) did not find a hyperplasia of the marrow as a whole but rather a slightly hypoplastic marrow. There was no resemblance to a leucemic marrow. The cellular marrow pattern as a whole was not greatly disturbed, but there were plasma cells and monocytoids, the latter of which were less like normal monocytes than were the similar elements that are found in the peripheral blood in this disease. The cytoplasm of the monocytoid cells varied from a marked basophilic to that which had no affinity for dyes, and the nuclear characteristics varied from the most immature, with nucleoli, to the most bizarre and extraordinary convolution. Piney believes that there should not be any confusion between glandular fever and German measles, although in both diseases Türk and plasma cells are found in the blood. In German measles there is a great excess of plasma cells in the lymph nodes, whereas the sternal marrow contains few plasma cells. On the other hand, in glandular fever (infectious mononucleosis) the reverse is the case; the sternal marrow contains large numbers of plasma cells, while the lymph nodes contain many monocytoid elements but a few plasma cells.

Moeschlin[24] simultaneously examined the sternal bone marrow, lymph nodes, and spleen in three cases of infectious mononucleosis. He observed a moderate myeloid immaturity of the marrow. The lymphoid cells in the bone marrow varied between 12 and 17 per cent, and there was a plasma cellular reaction moderate in degree. Moeschlin believes that the lymphocytes are more probably derived from blood aspirated during the puncture than from the marrow itself. In the lymph node he observed lymphoid and monocytoid elements in mitosis as well as normal lymphocytes. The plasma cells observed in the lymph nodes in cases of infectious mononucleosis are considered to be different from those seen in the lymph nodes in rubella. In rubella, puncture of the lymph nodes reveals numerous large primitive cells with reticular nuclei containing nucleoli. These plasmoblasts were observed in various stages of maturity or transition to the typical plasma cell seen in the peripheral blood. In the lymph nodes the highest number of plasmoblasts were found on the second day of the illness, while in the blood they were found at their greatest peak on the fifth day. In the bone marrow neither the plasmoblasts nor plasma cells were found in any significant numbers. In one case, perhaps by chance, a lymph follicle in the bone marrow was punctured, and an increased number of plasma cells and plasmoblasts was observed. Moeschlin[25] believes that the lymph node and bone marrow plasma cells are totally distinct both in structure and origin.

Leitner[26] studied the sternal marrow in two cases of infectious mononucleosis and observed a marked myelocytic reaction. The lymphocytes averaged

5.6 per cent of the bone marrow differential count. He attributed the maturation arrest of the myeloid tissue in the bone marrow to hyperfunction of the spleen, and this action on the marrow results in a leucopenia and granulocytopenia in the peripheral blood.

Propp and Schwind[27] performed sternal punctures on two cases of infectious mononucleosis and reported on the findings in one of the cases. The marrow revealed a normal degree of cellularity and a normal distribution of cell types. There was a marked lymphocytosis, but no lymphoblasts were seen. The heterophile antibody test was negative. Propp and Schwind believe that because of the high percentage of lymphocytes in the peripheral blood in this disease a relatively high lymphocyte count will occur in the marrow, but the marrow is not being replaced by lymphocytes. They support the opinion that there are occasional small lymph nodules in normal bone marrow, and therefore an increase of lymphocytes is not necessarily of importance.

Owing to the extremely low mortality from infectious mononucleosis, there has been very little opportunity to examine the bone marrow from various sites histologically. Further, in the few cases that have come to autopsy, there has been a failure to study the bone marrow. Haken[28] reported on the autopsy findings in three children with supposedly infectious mononucleosis, but he neglected to examine the bone marrow. Koenigsberger,[29] in analyzing the cases reported by Haken, was of the opinion that they were not cases of monocytic angina but rather cases of malignant diphtheria. DuBois[30] reported a case of infectious mononucleosis in a 26-year-old man which was complicated by pneumonia and empyema and at autopsy revealed a hyperplasia of the reticuloendothelial system. The bone marrow showed a practically normal erythroblastic series except for a few caryorrhetic normoblasts. The megakaryocytes, especially the immature forms, were reduced in number. The younger cells of the neutrophilic series were decreased in number at the expense of large mononuclear cells which had a cytoplasm that was basophilic and vacuolated. These cells appeared to be a part of a markedly active reticulum. There is no definite proof that this is a genuine case of infectious mononucleosis.

Ziegler[31] reported on the autopsy findings in a 22-year-old white woman with infectious mononucleosis with a heterophile antibody test of 1:640. At one time during the course of the disease, the leucocyte count was 23,800 per cubic millimeter with 93 per cent mononuclear cells in the peripheral blood. The lesions and changes in the liver, spleen, lungs, and kidneys were described, but unfortunately the bone marrow was not examined.

MORPHOLOGY OF THE LYMPHOCYTES IN INFECTIOUS MONONUCLEOSIS (DOWNEY[3])

Three types of lymphocytes, depending entirely on their morphologic features, give the blood its characteristic appearance in infectious mononucleosis. In the type I nucleus the chromatin forms a coarse network of heavy strands and masses which are not sharply separated from the parachromatin. In some of the larger cells there may be a slight tendency toward a more diffuse arrangement of the chromatin, and the lymphocytic nature of the nucleus always remains evident. The nuclei of the larger cells are frequently placed eccentrically

and are generally lobulated, indented, or kidney-shaped, and smaller cells also have irregular nuclei. The cytoplasm is quite characteristic. The degree of basophilia varies, but most of the cells are very basophilic. With Wright's stain the spongioplasm appears as dark blue or slate blue, finely granular, and of flaky material with a pale yellowish background of hyaloplasm. Its distribution

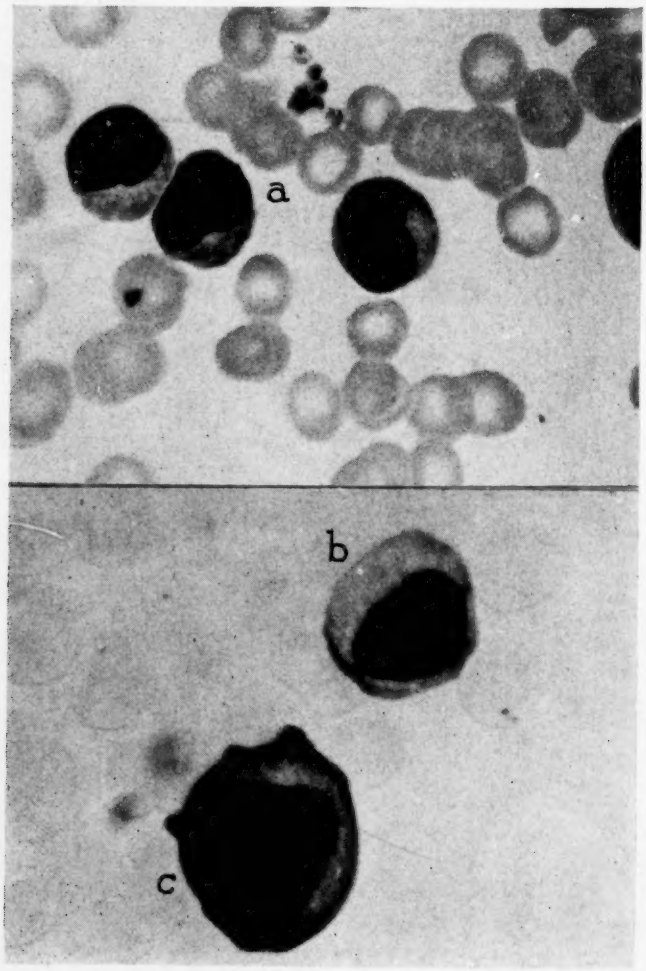

Fig. 1.—Peripheral blood films from two cases of infectious mononucleosis. *a*, Lymphocytes Downey type I. These lymphocytes were the type commonly seen in our cases. *b*, Lymphocyte Downey type III with a mature type of nucleus. *c*, Type III cell with an immature type of nucleus.

gives the cytoplasm a vacuolated, foamy, or mottled appearance. The peripheral portion may contain relatively more spongioplasm and, therefore, appear darker and more homogenous than other parts of the cytoplasm. If the nucleus is indented, the indentation is lighter than the rest of the cell because it contains relatively more of the yellowish hyaloplasm. Frequently, there are one or more

azurophilic granules embedded in the hyaloplasm of this region, which give the whole structure the appearance of a centrosphere. Most of the atypical cells contain azurophile granules, being quite abundant in most of the larger cells, and are of the lymphocytic type, that is, fine and carmine red.

In an atypical cell of type II the nucleus is somewhat similar to that of a plasma cell. The chromatin strands are very coarse, and there are several dense, rounded, or angular masses of chromatin among them. They have more of a washed appearance, and the blocking of the chromatin is not so pronounced as in the plasma cell. The cytoplasm has fewer vacuoles, and its spongioplasm has a smoother appearance which does not give the foamy, spongy, and stippled effect noted in type I. It is generally less basophilic, and there is a more even mixing of the basophilic portion of spongioplasm and hyaloplasm. In some cells there is the tendency of the basophilic portion of the cytoplasm to be arranged in the form of broad bands radiating from the nucleus. The cell body is generally wider than in the cells of type I, and the nucleus is rarely lobulated.

Type III cells have more leucemic features than those of types I and II. The cytoplasm is vacuolated and in some cells is quite basophilic. In the larger cells there is relatively more hyaloplasm, but the distribution of the spongioplasm remains about the same as in type I. These cells may contain a single large vacuole with an azurophile rod and within an indentation of the nucleus in addition to smaller vacuoles distributed rather evenly throughout the cytoplasm. The nucleus may be close to that of a stem cell. There may be a diffuse, vacuolated nucleus containing nucleoli (Fig. 1).

METHODS AND MATERIAL

Twenty-five patients with infectious mononucleosis, of whom eighteen were men and seven were women, comprised the group studied. No Negroes were observed. Patients varied in age from 13 months to 35 years. Fourteen patients ranged in age from 16 to 26 years, four were less than 16 years of age, and one was over 35 years of age. Many of the patients were medical and dental students referred to the University of Illinois, and the remainder were seen in consultation because of some clinical or hematologic finding observed by the medical examiner while performing a physical examination (lymphadenopathy or splenomegaly) or by the technician while doing a differential blood count. The heterophile antibody reaction was positive in thirteen, negative in six, and the test was not performed in the remaining patients. A false-positive Wassermann reaction was not observed.

The method of sternal aspiration and the preparation of the marrow for study has been reported in detail by one of us (L. R. L.).[32]

After films of the bone marrow were prepared the slide was air dried for several hours and stained with May-Grunwald-Giemsa or Wright stain and studied microscopically for cell distribution and types. When the stained films of the bone marrow were studied the ratio of myeloid to erythroid cells, the dispersion of the nucleated red cell series, the dispersion of the myeloid cell series, and the number of megakaryocytes were recorded. In recording the erythropoietic elements, the terms pronormoblast, basophilic, polychromatophilic, and

orthochromatic (acidophilic) normoblast were used in order of increasing maturity. Similarly, for the granulopoietic series, myeloblast, leucoblast, promyelocyte, myelocyte, metamyelocyte, and polymorphonuclear neutrophile were used. The lymphocytes which include both normal and atypical or immature forms were recorded together and expressed as the number of lymphocytes per 100 nucleated marrow cells. Plasma cells, monocytes, and reticuloendothelial elements were noted and described as a part of the bone marrow pattern when present in greater numbers than normal, but they were not included in the counts used to determine the above percentage relationships.

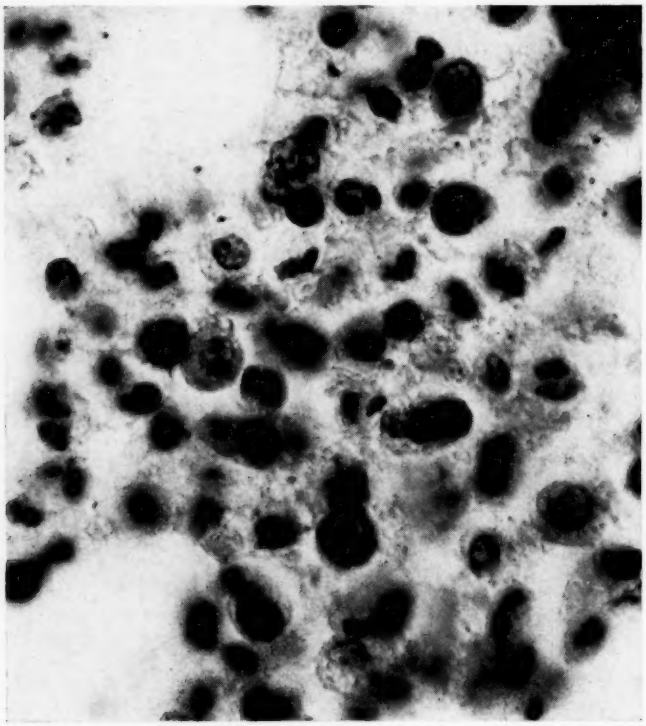

Fig. 2.—Bone marrow (histologic specimen) from a case of infectious mononucleosis showing a neutrophilic myelocytic pattern with absence of lymphocytic infiltration. Many of the lymphocytes in the peripheral blood were Downey type III. Heterophile antibody titer was 1:1224.

In a few cases of infectious mononucelosis histologic studies of the bone marrow were made from "hemopoietic particles" or "floaters" of the marrow obtained by sternal aspiration or biopsy material obtained by means of a small trephine. The marrow specimen was immediately placed in Zenker-acetic acid solution, and the sections after fixation were prepared in the usual way with paraffin and stained with eosin-azure or Giemsa. The sections were utilized in studying the topographic relationships of the marrow (Fig. 2).

Immediately preceding or following the marrow aspiration, an erythrocyte and leucocyte count, a hemoglobin determination (Newcomer's method) differen-

tial count, and a reticulocyte and platelet estimation were made with standardized and certified materials and apparatus. A hematocrit reading, sedimentation rate, and icterus index were done by the Wintrobe method.[33] Mean corpuscular volume, hemoglobin, and hemoglobin concentration were then computed.

DISCUSSION OF RESULTS AND ANALYSIS

Peripheral Blood.—Of the twenty-five patients with infectious mononucleosis, four had an anemia; in three the anemia was normocytic in type and in one, an infant 13 months of age, it was microcytic and hypochromic in character. All four patients had a positive heterophile antibody test. Except for one with a hemoglobin value of 8.5 Gm., all patients had a hemoglobin reading of 11.0 Gm. or more. In five the hemoglobin value was less than 13.5 Gm., and the highest value was 16.5 grams. The hemoglobin reading in all the patients studied ranged between 8.5 to 16.5 Gm., with an average of 13.54 grams. The mean corpuscular volume and mean corpuscular hemoglobin concentration were normal in all cases, except in the one with the microcytic and hypochromic anemia. The heterophile antibody test was positive in this instance.

The lowest erythrocyte count was 3,100,000 and the highest 6,560,000 per cubic millimeter, with an average of 4,660,000. In nine cases it was less than 4,500,000 and in only two cases was the erythrocyte value less than 4,000,000 per cubic millimeter.

The leucocyte count varied between 4,500 and 22,000 per cubic millimeter, with an average count of 10,900. In ten cases the count was less than 10,000, and in two of these cases the white count was below 5,000. In only one case was the count over 20,000. The characteristic finding in the differential count was the large number of abnormal lymphocytes which ranged from 37 to 82 per cent, with an average of 64 per cent. The atypical "leucocytoid" lymphocytes were of the type I and II of Downey[3] with various degrees of variation between the types.

Extremely immature cells of the type III of Downey were rarely found in our series of cases of infectious mononucleosis. In only one case which was complicated by pulmonary tuberculosis were so-called "lymphoblasts" observed; that is, cells whose nuclear structure indicated immaturity or lacked differentiation. Some of the latter cells approached the Türk irritation form of plasma cells with diffuse myeloblastic nucleus (leptochromatic). Lymphocytic plasma cells were seen in all of the other cases as well as normal lymphocytes (mostly small types). The typical lymphoblast of acute and subacute lymphatic leucemia was not seen in our cases. Degenerated and fragile cells were absent from the blood films.

The granulocytes varied from 3 to 5 per cent. Moderate to marked toxic neutrophiles with a moderate shift to the left in the neutrophilic picture were seen in all cases. The shift to the left consisted in increased numbers of nonfilament granulocytes, the so-called "stab" forms, which ranged over 6 per cent in twelve cases and over 25 per cent in three cases. Neutrophilic metamyelocytes were observed in two cases, 1 per cent in the first and 2 per cent in the second

case. Myelocytes, promyelocytes, and myeloblasts were not seen. Basophilic granules or "toxic" granules, as they are often designated, were seen in the granulocytes in all the cases. The grade 3 to 4 "toxic" granulations consisted of small and large types of granules which in many instances filled the entire cytoplasm of the neutrophile. Oxidase stain on such cells revealed relatively few oxidase reacting granules (normal neutrophilic granules). Degenerative changes of the nucleus, such as condensation in different areas, were occasionally found, but fragmentation of the nucleus, swelling of the cytoplasm with vacuole formation, diffuse basophilic staining in the cytoplasm, and marked irregularity in size of the neutrophiles showing "toxic" and degenerative changes were only rarely observed.

The monocytes which were over 8 per cent in four cases were morphologically normal. The normal appearing eosinophiles were definitely increased in only one case with a value of 16 per cent. This case with a positive heterophile antibody reactive had 37 per cent atypical lymphoids and 38 stab cells in the peripheral blood at the time that the bone marrow was studied. There were only 7 per cent neutrophiles in the differential count. The basophiles were over 1.0 per cent in three cases; the highest count was 4.0 per cent in one case.

The platelets were normal in number and morphology in practically all cases and moderately increased in two cases from blood film examination.

The corrected sedimentation rate was increased in sixteen cases with values as high as 41 mm. per hour.

The reticulocyte count was normal in all cases. The icterus index was higher than 7.5 units in three cases, and in one of the cases with a value of 15 units there was a clinical jaundice.

Bone Marrow.—The bone marrow was hyperplastic in twenty cases with volumetric values as high as 35 per cent and with an average reading for all cases of 14.4 per cent. This is more than twice the normal value. The bone marrow cell counts ranged from 210,000 to 670,000 per cubic millimeter in eighteen cases, with an average count of 430,000 per cubic millimeter which is approximately one and one-half times the normal value (300,000).

The fat volume (volumetric reading) averaged 1 per cent, with the highest reading of 5 per cent in a 12-year-old boy.

The myeloid elements besides being increased in number revealed a moderate to marked degree of granulopoietic immaturity. Immaturity was never carried to the stage of myeloblastic involvement. The greatest increase occurred in the neutrophilic myelocytes which in some cases comprised over 50 per cent of the granulopoietic series. This is a marked increase when compared with the normal value of 10.8 per cent. In eighteen cases the neutrophilic myelocytes ranged over 15 per cent, and in only three cases were they less than 10 per cent. Moderate to marked degrees of "toxic" granules were observed in many of the neutrophilic myelocytes, even in phases of mitotic division (Fig. 3). The "toxic" or coarse basophilic granules which may fill the cells are different from the heavy, wine-colored or reddish lavender granulation observed in normal maturing neutrophilic myelocytes. The delicate neutral- or lilac-colored granules

which are observed in the Hof of the nucleus in many normal maturing neutrophilic myelocytes were absent, and the normal blue color seen in some of the early neutrophilic myelocytes was occasionally replaced by a diffuse basophilic staining cytoplasm. Marked degenerative changes of the nucleus or cytoplasm with the formation of the vacuoles were rarely observed. In cases of infectious mononucleosis with a predominating neutrophilic metamyelocytic type of bone marrow, the metamyelocytes were practically free of any toxic granules or degenerative changes, while the myelocytes showed the morphologic effect of a toxic process. These findings indicate that the "degenerative" shift to the left

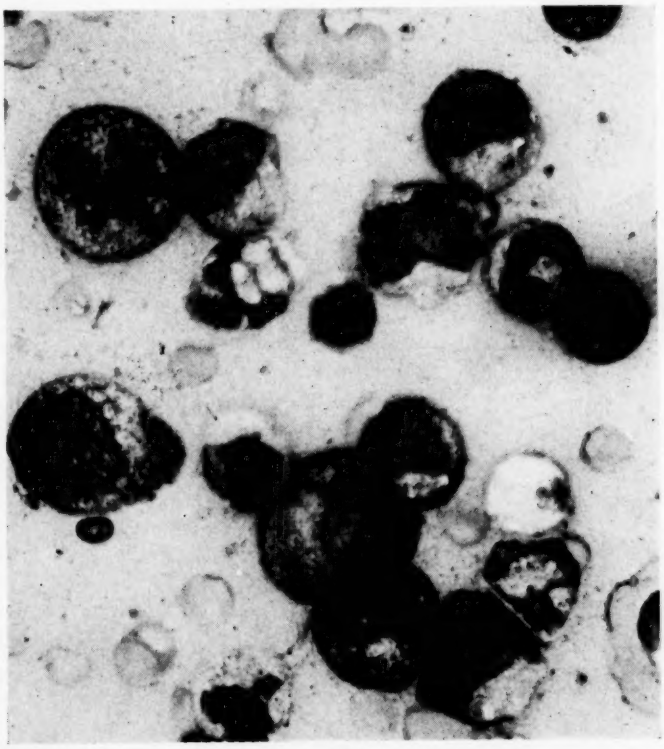

Fig. 3.—Bone marrow (concentration technique) from a case of infectious mononucleosis showing a severe toxic condition of the granulopoietic elements. Note the toxic neutrophilic myelocyte in phase of mitosis and the absence of lymphocytes in the bone marrow.

observed in the peripheral blood with an increased number of immature forms ("stab-kernige" or staff forms[34]), in which the nuclei are narrow, deeply staining with little or no structural detail, but more often with few or many small or large basophilic granules (toxic) in a diffuse basophilic staining cytoplasm, is the effect of toxemia on the development of the cell at the early neutrophilic myelocytic stage; that is, during the stage of specific granule formation and the beginning of nuclear configuration. In infectious mononucleosis the toxemia affects the cytoplasmic granules to a much greater extent than the nucleus, since many of the cells of the myelocytic series with toxic granules are observed in mitosis. There is no definite indication of a depressed bone marrow function

to any marked degree. The toxemia of infectious mononucleosis affects the granulopoietic tissue in the bone marrow at the early neutrophilic myelocyte stage of development to prevent the differentiation to neutrophiles, so that they develop to a certain point and emerge into the peripheral blood as stab forms with varying degrees of toxic granules in the cytoplasm. Apparently those myelocytes that escape the toxic process complete their development through the several stages of neutrophilic metamyelocyte formation and become normal polymorphonuclear neutrophiles (Fig. 4). Therefore, the neutrophilic metamyelocytes in the bone marrow in cases of infectious mononucleosis probably do not take any active part in the formation of the toxic neutrophiles (stab forms) seen in the peripheral blood in this disease.

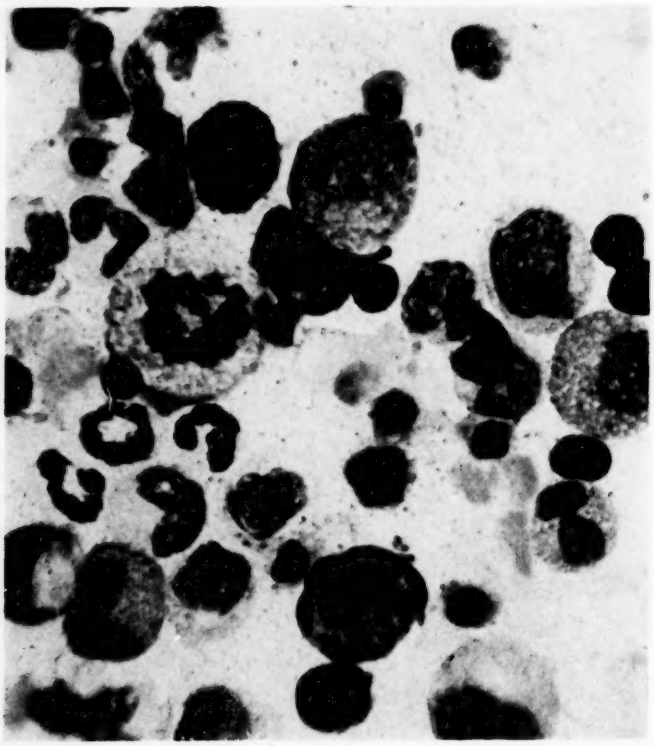

Fig. 4.—Bone marrow (concentration technique) from a case of infectious mononucleosis showing neutrophilic myelocytes and neutrophilic metamyelocytes; the latter reveal normal development with an absence of toxic granules in the cytoplasm. Note the absence of lymphocytes in the bone marrow. Heterophile antibody titer was 1:1792.

Except for an increased number of eosinophilic myelocytes and metamyelocytes in the bone marrow in one case which was reflected by an eosinophilia (mature cells) in the peripheral blood of 16 per cent, all the other marrows showed a normal number of eosinophiles. The basophiles were reduced in number. The mature neutrophiles averaged 7.9 per cent of the marrow granulocytes. This is approximately one-third the number seen in normal bone marrows and is due in a large part to the increase in the number of neutrophilic myelocytes.

The erythroid tissue was normal, except in the patient with the microcytic hypochromic anemia in whom the bone marrow revealed a normoblastic hyperplasia. In several of the marrows, groups or clusters of normoblastic elements were observed which is indicative of erythroid stimulation.

The megakaryocytes were increased in number in fifteen cases and normal in number in ten cases of infectious mononucleosis. The megakaryocytic increase was pronounced in those marrows with a myeloid hyperplasia. The maturation dispersion of the megakaryocytes was normal.

In a case of infectious mononucleosis in a girl 26 years of age with diabetes mellitus and not included in this group of twenty-five cases, the histiocytes or reticulum cells were increased in number and normoblastic clusters with frequent mitotic cells were seen scattered among the hyperplastic myeloid tissue. The reticulum cells appeared morphologically normal, and there were no indications that these elements were developing into specific hemopoietic elements such as myeloid, erythroid, megakaryocytes, plasma cells, and normal or abnormal lymphoid cells.

Plasma cells were only rarely seen in the marrow in our cases of infectious mononucleosis. In a 25-year-old woman with pulmonary tuberculosis who had immature lymphoid cells with basophilic cytoplasm in the peripheral blood, the bone marrow contained an increased number of normal and atypical types of plasmacytoid cells, some of which approached the Türk type with a leptochromatic structure of the nucleus. Cells of this type were also seen in the peripheral blood.

The number of lymphoid cells ranged between 4 to 24 per 100 bone marrow cells with an average of 10 per cent for the twenty-five cases. The greater number of lymphoid cells was seen in those marrows in which there was a lack of myeloid hyperplasia and in which admixture with peripheral blood was more pronounced than normal as indicated by the increased number of mature neutrophiles. This latter finding with an increased number of lymphoid cells is always a definite indication of peripheral blood dilution and means that the bone marrow sample is only partially representative of the bone marrow pattern. The single or groups of lymphocytes seen in the bone marrow were similar to those seen in the peripheral blood. There were no morphologic indications from either the nucleus or cytoplasm that any of the marrow elements including those of the histiocytic system (reticulum cells) were giving rise to the normal or atypical lymphoid elements. In the marrows with a marked myeloid hyperplasia, the lymphocytes were decreased in number when compared with normal marrows in which the average number of lymphocytes is 17.8 per 100 bone marrow cells with a range of 12 to 26 per cent.

The myeloid-erythroid ratio averaged 3.0:1.0 with a reversal of the ratio in the marrow with the normoblastic hyperplasia (1.1:1.0) and a microcytic and hypochromic anemia in the peripheral blood; there was an increased myeloid ratio (6.69:1) in those marrows with a granulopoietic hyperplasia. The normal myeloid-erythroid ratio in our control cases was 2.75:1.0.

TABLE I. FINDINGS IN BLOOD IN INFECTIOUS MONONUCLEOSIS, NORMALS, CHRONIC LYMPHOCYTOSIS, WHOOPING COUGH, AND MEASLES

	HEMOGLOBIN (GM./100 C.C. BLOOD)	ERYTHROCYTES (MILLIONS)	LEUCOCYTES (THOUSANDS)	PLATELETS (THOUSANDS)	HEMATOCRIT (ERYTHROCYTES) (%)	HEMATOCRIT (LEUCOCYTES) (%)	SEDIMENTATION RATE (UNCORRECTED) (MM./HOUR)	SEDIMENTATION RATE (CORRECTED) (MM./HOUR)	MEAN CORPUSCULAR VOLUME (C. MM.)	MEAN CORPUSCULAR HEMOGLOBIN (MICROMICROGRAMS) (μμ)	MEAN CORPUSCULAR HEMOGLOBIN CONCENTRATION (%)	RETICULOCYTE COUNT (%)	ICTERUS INDEX (UNITS)	NEUTROPHILIC METAMYELOCYTES (%)	STAB FORMS (NEUTROPHILES) (%)	POLYMORPHONUCLEAR NEUTROPHILES (%)	LYMPHOCYTES (%)	MONOCYTES (%)	EOSINOPHILES (%)	BASOPHILES (%)
Infectious mononucleosis (average, 25 cases)	13.54	4.66	10.9	Normal	41.3	0.75	26.1	20.4	86.8	29.5	33.2	0.7	6.8	0.12	8.52	19.3	64.0	5.86	1.48	0.72
Range	8.5	3.10	4.5	Normal	32.0	0.5	4.0	2.0	49.0	13.0	26.0	0.3	3.0	0.0	0.0	3.0	37.0	0.0	0.0	0.0
	16.5	6.56	22.2	to slight increase	49.5	1.5	64.0	42.0	98.0	36.0	37.0	1.2	15.0	2.0	38.0	50.0	82.0	12.0	16.0	4.0
Normals (10*)	14.9	5.0	7.45	250.0 to 350.0	44.6	0.5	10.5	7.3	88.5	29.5	33.5	0.3	6.5	0.0	5.1	54.5	29.5	8.9	1.4	0.6
Chronic lymphocytosis (2)	12.45	3.98	19.22	Normal	36.7	1.0	38.5	19.5	93.0	31.0	33.5	2.5	6.2	0.0	38.5	58.0	0.5	2.0	1.0	0.0
Whooping cough (1)	10.5	4.37	17.85	Normal	32.0	1.0	28.0	5.0	73.0	24.0	32.0	0.3	5.0	0.0	1.0	5.0	86.0	6.0	1.0	1.0
Measles (1)	16.5	5.56	7.1	Normal	52.0	0.5	13.0	12.0	93.0	29.0	31.0	1.0	5.0	0.0	35.0	26.0	24.0	11.0	2.0	2.0

*Number of cases studied.

TABLE II. BONE MARROW FINDINGS IN INFECTIOUS MONONUCLEOSIS, NORMALS, CHRONIC LYMPHOCYTOSIS, WHOOPING COUGH, AND MEASLES

	MYELOID-ERYTHROID VOLUME (%)	FAT VOLUME (%)	MEGAKARYOCYTES (PER 18 MM. SQUARE)	NUCLEATED MARROW CELLS (THOUSANDS PER C.MM.)	MYELOBLASTS (%)	PROMYELOCYTES* (%)	NEUTROPHILIC MYELOCYTES (%)	EOSINOPHILIC MYELOCYTES (%)	NEUTROPHILIC METAMYELOCYTES (%)	EOSINOPHILIC METAMYELOCYTES (%)	POLYMORPHONUCLEAR NEUTROPHILES (%)	EOSINOPHILES (%)	BASOPHILES (%)	PRONORMOBLASTS (%)	BASOPHILIC NORMOBLASTS (%)	POLYCHROMATOPHILIC NORMOBLASTS (%)	ORTHOCHROMATIC (ACIDOPHILIC) NORMOBLASTS (%)	MYELOID-ERYTHROID RATIO (% AND RATIO)	LYMPHOID CELLS IN MARROW (PER 100 MARROW CELLS)
Infectious mononucleosis (average 25 cases)	14.4	1.0	Normal increase	4.30	0.44	1.10	24.8	1.96	61.34	1.84	7.4	1.04	.08	2.1	8.32	87.58	2.0	75:25 3:1	10.0
Range	5.0	0.0	Normal	210.0	0.0	0.0	6.0	0.0	32.0	0.0	2.0	0.0	0.0	1.0	2.0	76.0	0.0	56:44 (1.3:1)	4.0
	35.0	5.0	increase	670.0 (18 cases)	3.0	4.0	53.0	10.0	84.0	7.0	26.0	3.0	2.0	10.0	17.0	96.0	7.0	87.13 (6.69:1)	24.0
Normals (10†)	6.8	3.2	52.2	300.0	0.4	0.9	10.8	2.0	57.8	3.0	23.1	1.5	0.5	2.6	13.2	78.3	5.9	73.35: 26.65 (2.75:1)	17.8 (12 to 26)
Chronic lymphocytosis (2)	5.5	3.5	Normal	190,000	0.5	1.0	19.5	5.0	59.5 13.5	3.0	9.0	2.0	0.5	5.0		79.5	2.0	62.5:37.5 6:1	22.0
Whooping cough (1)	8.0	0.0	Normal	410.0	1.0	1.0	35.0	1.0	51.0	5.0	5.0	1.0	0.0	1.0	4.0	94.0	1.0	85:15 5.6:1	20.0
Measles (1)	5.0	2.0	Increase	230.0	0.0	1.0	25.0	3.0	63.0	1.0	4.0	2.0	0.0	1.0	14.0	85.0	0.0	65:35 (1.85:1)	5.0

*Includes leucoblasts.
†Number of cases studied.

TABLE III. SUMMARY OF CONDITIONS STUDIED

	PERIPHERAL BLOOD		BONE MARROW STUDIES				
	LEUCOCYTES (THOUSANDS)	LYMPHOID AND "BLAST" CELLS (%)	MYELOID-ERYTHROID VOLUME (%)	CELL COUNT THOUSANDS)	MYELOCYTES) (%)	NEUTROPHILES (%)	LYMPHOID AND "BLAST" CELLS (%)
Normal controls (10*)	7.45	29.5	6.8	300.0	10.8	23.1	17.8
Infectious mononucleosis (25)	10.9	64.0	14.4	430.0	24.8	7.9	10.0
Whooping cough (1)	17.85	86.0	12.0	410.0	35.0	5.0	20.0
German measles (Rubella) (1)	7.1	24.0	5.0	230.0	8.0	4.0	5.0
Chronic lymphocytosis (2)	28.5	54.5	5.5		19.5	11.0	22.0
Chronic lymphatic leucemia (14)	14.2	77.0	Hyperplastic				50 and higher
Acute and subacute lymphatic leucemia (24)	11.67	39.0	Hyperplastic				Marked (over 90)
Acute leucemia (atypical types) (8)	10.4	50.0	Hyperplastic				Marked (over 90)

*Number of cases studied.

Besides the "normal" controls, the blood and bone marrow was studied in cases of whooping cough, German measles, chronic nonleucemic lymphocytosis, chronic lymphatic leucemia, acute and subacute lymphatic leucemia, and acute "atypical" types of leucemia. These findings are recorded in Tables I to III.

CHRONIC NONINFECTIOUS LYMPHOCYTOSIS

Three cases of chronic lymphocytosis were studied in two men, one 46 and the other 48 years of age, and a woman 42 years of age. The average blood and bone marrow studies in the men are recorded in the tables. The woman presented similar clinical and hematologic findings. In these cases it was noted during a routine blood examination that a lymphocytosis consisting of normal small lymphocytes comprised a large part of the differential count. There was no lymphadenopathy nor splenomegaly. The bone marrow of the woman patient studied showed a slight hypoplasia, quantitatively, with a myeloid-erythroid volume of 3 per cent. The myeloid, erythroid, and megakaryocytic elements showed all the phases of normal development, and 50 lymphocytes were counted among 100 nucleated bone marrow cells. The two cases recorded in the tables have been observed for over seven years and have not showed any clinical or hematologic changes. It is difficult to classify these cases. They may represent early cases of chronic lymphatic leucopenic leucemia or cases of chronic benign lymphocytosis. Only continued clinical and hematologic observations will finally reveal the diagnosis.

WHOOPING COUGH

A case of whooping cough in a boy 6 years of age was studied hematologically. The findings in the blood and bone marrow are recorded in the table, and it will be noted the anemia is of a simple microcytic type. In spite of the marked lymphocytosis in the peripheral blood, the bone marrow reveals approximately the same number of lymphocytes found in the normal bone marrow.

The erythroid and megakaryocytic tissues were normal and myelopoiesis was of a toxic type.

GERMAN MEASLES (RUBELLA)

In a case of German measles in a medical student 26 years of age, the peripheral blood studies with the exception of the blood film were normal. The marked toxic shift to the left in the Schilling index with the presence of 6 plasma cells among 100 leucocytes was the most significant abnormality in the peripheral blood. With the exception of a slight increase in the number of megakaryocytes and a moderate toxic type of granulopoiesis, the bone marrow was normal. The number of lymphocytes in the bone marrow was decreased. No plasma cells were observed in the marrow in spite of these frequent findings in the peripheral blood. The significance of plasma cells in various infective conditions has been previously noted.[35]

LEUCEMIA

Forty-six cases of leucemia were studied as controls (see Table III). In chronic lymphatic leucemia, irrespective of the quantitative or qualitative lymphocyte count in the peripheral blood, the bone marrow revealed a moderate to marked lymphocytic replacement of the bone marrow, depending upon the stage and duration of the disease. In the subacute and acute cases of lymphatic leucemia as well as stem cell, myeloid, monocytic, and atypical types, the bone marrow was always filled with proliferative types of blast cells which replaced the normal bone marrow elements (Fig. 5). This resulted in the neutropenia, anemia, and thrombopenia in the peripheral blood. The findings in the marrow were observed whether the leucocyte count in the peripheral blood was normal, subleucemic (leucopenic), or increased.

DISCUSSION

The diagnosis of infectious mononucleosis is based on three essential factors: (1) the clinical picture, (2) the serologic reaction, and (3) the hematologic studies, especially cytologic study of the blood. The presence of a relative large number of atypical "leucocytoid" lymphocytes is the most constant and, therefore, the most useful diagnostic sign of the disease.

Anemia does not occur in uncomplicated cases. The finding of a normocytic anemia in several of our cases and the presence of an iron deficiency anemia in one of our cases in all probability existed prior to the infectious mononucleosis. The toxic process associated with the disease is of too short duration to produce a toxic anemia. The study of the erythroid elements in the marrow of our cases with anemia was that seen in long-standing anemia states. In some of the cases without an anemia in the peripheral blood, a few clusters of normoblastic tissue were seen which was indicative of an erythropoietic response to an acute toxic condition. These normoblastic clusters in the bone marrow were of infrequent occurrence, in contrast to the frequent and widespread areas of erythropoietic activity seen in chronic anemia. The platelet count was normal

in most of the cases and moderately increased in several of the other cases of infectious mononucleosis. A low platelet count was not seen in our cases, and the bone marrow megakaryocytes were normal or increased in number with normal morphologic characteristics.

The abnormal cells which characterize the disease have been previously described. It is generally agreed that the cell is a benign type of lymphocyte which has its origin from the reticulum of the lymphatic tissue anywhere in the body. Apparently, the reticulum of the bone marrow does not take any active part in the formation of the atypical lymphocytes seen in this disease. It may be

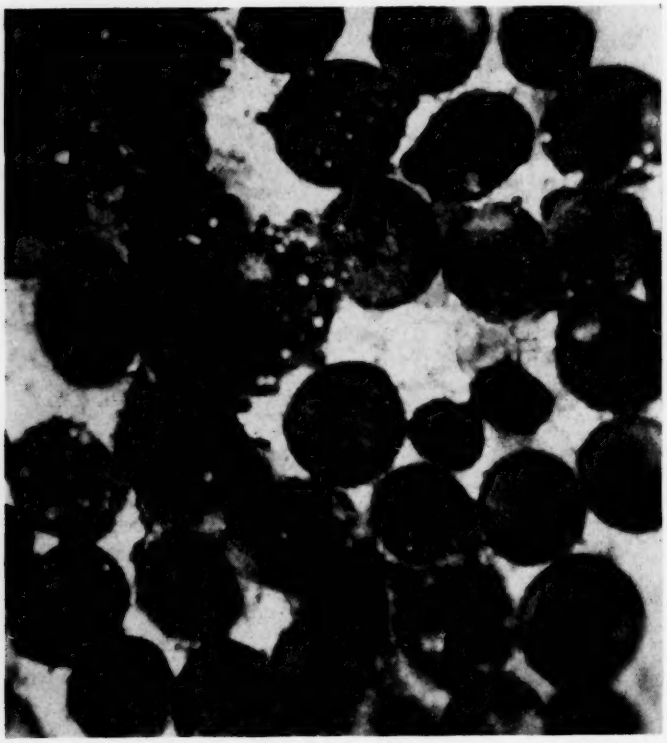

Fig. 5.—Bone marrow (concentration technique) from a case of acute leucopenic myelosis showing the marrow filled with myeloblasts which have replaced the granulopoietic, erythropoietic, and megakaryocytic tissue.

that the products of the proliferative granulocytic cells in the bone marrow act as a protective mechanism during the toxic and neutropenic phase of the disease. Although little is known concerning the function and the potentialities of abnormal lymphocytes seen in the blood in cases of glandular fever, they lack the metastatic or replacement characteristics of the pathologic lymphocytes seen in the malignant lymphomas and leucemia. In infectious mononucleosis which is associated with a large number of atypical lymphocytes in the peripheral blood, the bone marrow shows a lack of lymphocytic infiltration and replacement of the marrow by lymphocytes seen in cases of lymphatic leucemia.

A number of cases of infectious lymphocytosis[36] have been reported in which there was a marked total leucocytosis with a high relative lymphocytosis and without clinical signs or symptoms. Leucocyte counts as high as 120,000 and differential counts with 97 per cent lymphocytes occurring in children have been reported. In several cases the bone marrow revealed many lymphocytes. There is no assurance that the presence of the lymphocytes in the bone marrow was not due to admixture with peripheral blood. The peripheral blood in these cases may resemble that seen in chronic lymphatic leucemia, but the bone marrow lacks the replacement or infiltration characteristic of lymphatic leucemia.

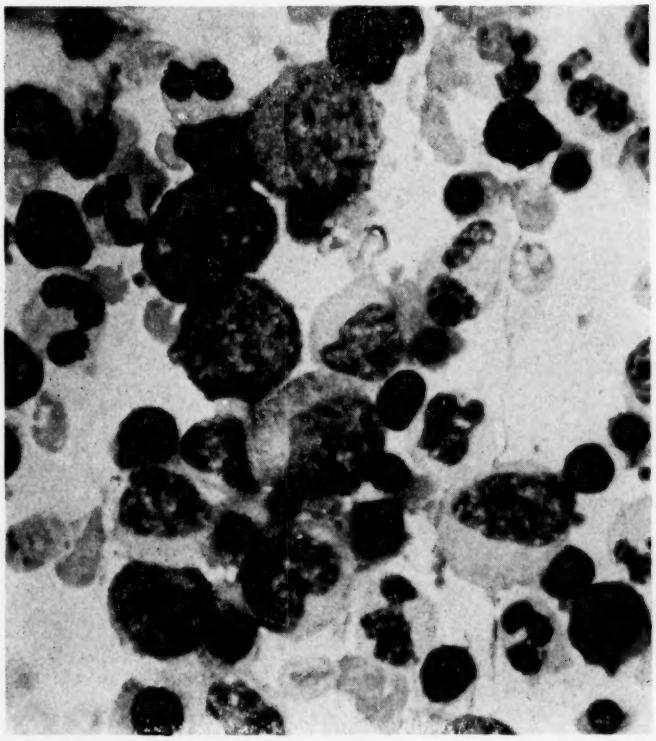

Fig. 6.—Bone marrow (concentration technique) from a case of acute infectious lymphocytosis showing moderate myeloid immaturity, normoblastic activity, and a few mature lymphocytes. The leucocyte count in the peripheral blood was 60,000 with a differential count consisting of 80 per cent small normal lymphocytes. Heterophile antibody reaction was negative.

In several of these cases studied by Limarzi and Poncher,[37] the bone marrow was hyperplastic and resembled the marrow of infectious mononucleosis (Fig. 6). Thus, bone marrow studies will immediately separate a malignant lymphocytic condition from a benign or nonleucemic reaction. It is of interest that the lymphocytes observed in the acute and chronic benign lymphocytosis do not possess the potentialities of transforming into macrophages as observed by Kolouch[38] in his experimental work on the lymphocytes in acute inflammation. Apparently, normal lymphocytes and atypical lymphocytes seen in lymphocytic reactions possess none of the malignant or transformation potentialities of

either leucemic cells or lymphocytes seen in acute inflammation; yet, leucemic and nonleucemic lymphocytes may appear morphologically similar. The pathologic, physiologic, and potential differences between benign and malignant types of lymphocytic elements are of interest in view of the recent work of Stedman[39] on the chemistry of the cell nuclei and the studies of Ehrich and Harris[40] on the role of the lymphocytes in relationship to antibody formation. Malignancy or malignant cells produced by very diverse causes differ from the normal chiefly, if not entirely, by the acquisition of the capacity for uncontrolled growth. According to Stedman any agent which interferes with the chemical nature of the nucleus, that is, which causes the histone content of the nucleus of a cell to fall below a certain unknown level, will render that cell malignant unless some mechanism is present to restore histone to the nucleus. Whether this line of investigation can be carried to explain the pathologic physiologic differences between normal lymphocytes, the atypical lymphocytes of infectious mononucleosis, and the malignant lymphocytes of leucemia is an interesting speculation. It has been suggested by the work of Ehrich and Harris[40] that the macrophages, the granular leucocytes, and the normal lymphocytes should be regarded as a team of substantially equal importance whose coordinate activities are essential for effective defense against infection. The granulopenia and the presence of atypical lymphocytes which may lack the power of formation of antibodies which are present in normal lymphocytes[41] and protect the body from infection may be the cause of the toxic reaction seen in infectious mononucleosis. The toxic agent would then act on the bone marrow to produce a so-called maturation arrest and resultant myeloid hyperplasia; or the suppression of their delivery (release mechanism) to the peripheral blood results in a myeloid hyperplasia.

CONCLUSIONS

1. In spite of the large number of atypical lymphocytes in the peripheral blood in infectious mononucleosis the bone marrow is not involved.

2. The bone marrow in infectious mononucleosis reveals a myeloid hyperplasia and immaturity.

3. Either the "toxic agent" acts on the bone marrow to produce a myeloid hyperplasia and suppresses their delivery to the peripheral blood or the suppression of the myeloid tissue results in a myeloid hyperplasia.

4. The atypical lymphocytes in infectious mononucleosis show none of the metastatic or replacement characteristics of leucemia cells. In contrast, cases of leucemia with many abnormal cells in the peripheral blood, the bone marrow reveals varying degrees of replacement of the normal marrow by leucemic cells. The replacement characteristic of leucemic cells in lymphatic leucemia is seen in the bone marrow when the blood count is leucopenic, normal, or increased in type.

5. Sternal puncture is of diagnostic aid in differentiating benign and malignant types of lymphocytosis.

REFERENCES

1. Pfeiffer, E.: Das Drusenfieber, Jahrb. f. Kinderh. **29**: 257, 1889.
2. Paul, J. R., and Bunnell, W. W.: Presence of Heterophile Antibodies in Infectious Mononucleosis, Am. J. M. Sc. **183**: 90, 1932.
3. (a) Downey, H., and McKinlay, C. A.: Acute Lymphadenosis Compared With Acute Lymphatic Leukemia, Arch. Int. Med. **32**: 82, 1923.
   (b) Heck, F. J.: Infectious Mononucleosis. In Downey, Hal (editor): Handbook of Hematology, vol. 4, New York, 1938, Paul B. Hoeber, Inc., p. 2583.
4. Freeman, W.: Bone Marrow Studies in Glandular Fever (Infectious Mononucleosis), Am. J. Clin. Path. **6**: 185, 1936.
5. Read, J. T., and Helwig, F. C.: Infectious Mononucleosis; an Analysis of Three Hundred Cases With Three Characterized by Rare Hematologic Features, Arch. Int. Med. **75**: 376, 1945.
6. Young, R. H., and Osgood, E. E.: Sternal Marrow Aspirated During Life; Cytology in Health and in Disease, Arch. Int. Med. **55**: 186, 1935 (footnote).
7. Nordenson, N. C.: Studies on Bone Marrow From Sternal Puncture. From the IV Medical Service, St. Eriks Hospital, Stockholm, Sweden, 1935.
8. Markoff, N.: Die Beurteilung des Knochenmarks durch Sternalpunktion, Deutsches Arch. f. klin. Med. **179**: 113, 1936.
9. (a) Rohr, K.: Knochenmarksmorphologie des menschlichen Sternalpunktates, Berlin, 1937, Urban and Schwarzenberg.
   (b) Rohr, K.: Das menschliche Knochenmark, Leipzig, 1940, Georg Thieme.
10. Mallarmé, J.: Le myelogramme normal et pathologique par ponction sternale, Paris, 1938, Gaston Doin & Cie.
11. Weil, P. E., and Perlés, S.: La ponction sternale, Paris, 1938, Masson & Cie.
12. Schulten, H.: Die Sternalpunktion als diagnostische Methode, Leipzig, 1937, Georg Thieme.
13. Klima, R.: Sternalpunktion und Knochenmarksbild bei Blutkrankheiten, Berlin, 1938, Urban and Schwarzenberg.
14. Henning, N.: Sitzungsbericht der I. internationalen haematologischen Tagung, 1938. Quoted by Scott, R. B.: Sternal Puncture in the Diagnosis of Diseases of the Blood-Forming Organs, Quart. J. Med. **8**: 127, 1939.
15. Henning, N., and Keilhack, H.: Die Ergebnisse der Sternalpunktion, Ergebn. d. inn. Med. u. Kinderh. **56**: 372, 1936.
16. Ustvedt, H. J.: Infektis Mononukleose, Norsk mag. f. laegevidensk. **98**: 139, 1937.
17. Fieschi, A.: Semeiologia del midollo osseo: Studio di morfologia clinica. In Ferrata, A.: Biblioteca "Haematologica," vol. 6, 1938, Pavia: Tipografia già Cooperativa.
18. Scott, R. B.: Sternal Puncture in the Diagnosis of Diseases of the Blood Forming Organs, Quart. J. Med. **8**: 127, 1939.
19. Vogel, P., Erf, L. A., and Rosenthal, N.: Hematological Observations on Bone Marrow Obtained by Sternal Puncture, Am. J. Clin. Path. **7**: 436, 498, 1937.
20. Vogel, P., and Bassen, F. A.: Sternal Marrow of Children in Normal and Pathologic States, Am. J. Dis. Child. **57**: 245, 1939.
21. Morrison, M., and Samwich, A. A.: Clinico-Hematologic Evaluation of Bone Marrow Biopsies, Am. J. M. Sc. **198**: 758, 1939.
22. Mendell, T. H., Meranze, D. R., and Meranze, T.: The Clinical Value of Sternal Bone Marrow Puncture, Ann. Int. Med. **16**: 1180, 1942.
23. (a) Piney, A.: Sternal Puncture: A Method of Clinical and Cytological Investigation, London, 1941, William Heinemann, Ltd.
   (b) Piney, A., and Hamilton-Paterson, J. L.: Ibid., ed. 2, New York, 1944, Grune and Stratton.
24. Moeschlin, S.: Die Genese der Drüsenfieberzellen (Mononucleosis Infectiosa) an Hand von Drüsen-, Sternal- und Milzpunktaten, Deutsches Arch. f. klin. Med. **187**: 249, 1941.
25. Moeschlin, S.: Untersuchungen über Genese und Funktion der Blutplasmazellen anhand von Lymphhdrüsen- und Sternalpunktaten bei Rubeolen, Helvet. med. acta **7**: 227, 1940.
26. (a) Leitner, S. J.: Das Lymphoidzellige Drüsenfieber (Mononucleosis infectiosa) mit besonderer Berücksichtigung des Sternalmarkbefundes, Schweiz. med. Wchnschr. **70**: 117, 1940.
   (b) Leitner, S. J.: Die intravitale Knochenmarksuntersuchung, Basel, 1945, Benno Schwabe.
27. Propp, S., and Schwind, J. L.: Sternal Puncture as a Practical Diagnostic Procedure, Ann. Int. Med. **21**: 580, 1944.
28. Haken von: Monozytenanginen mit letalem Ausgang, Deutsche med. Wchnschr. **53**: 565, 1927.

29. Koenigsberger, E.: Monozytenangina oder maligne Diphtherie, Deutsche med. Wchnschr. **53**: 883, 1927.
30. DuBois, A. H.: De la pathogénie de l'angine à monocytes, Acta med. Scandinav. **73**: 237, 1930.
31. Ziegler, E. E.: Infectious Mononucleosis: Report of a Fatal Case With Autopsy, Arch. Path. **37**: 196, 1944.
32. Limarzi, L. R.: The Diagnostic Value of Sternal Marrow Aspirations, Ill. M. J. **75**: 38, 1939.
33. Wintrobe, M. M.: Anemia: Classification and Treatment on Basis of Differences in Average Volume and Hemoglobin Content of Red Corpuscles, Arch. Int. Med. **54**: 256, 1934.
34. (a) Schilling, V.: Ein Praktisch und zur Demonstration brauchbaren Differential Leukozytometer mit Arnethschen Verschiebund des Blutbildes, Deutsche med. Wchnschr. **37**: 1159, 1911.
(b) Haden, R. L.: Qualitative Changes in Neutrophilic Leukocytes, Am. J. Clin. Path. **5**: 354, 1935.
35. Hickling, R. A.: The Significance of Haemic Plasma Cells in Various Infective Conditions, J. Hyg. **24**: 120, 1925.
36. (a) Smith, C. H.: Infectious Lymphocytosis, Am. J. Dis. Child. **62**: 231, 1941.
(b) Duncan, P. A.: Acute Infectious Lymphocytosis, Am. J. Dis. Child. **66**: 267, 1943.
(c) Finucane, D. A., and Philips, R. S.: Infectious Lymphocytosis, Am. J. Dis. Child. **68**: 301, 1944.
(d) Birge, R. F., and Hill, L. F.: Acute Infectious Mononucleosis, Am. J. Clin. Path. **15**: 508, 1945.
37. Limarzi, L. R., and Poncher, H. C.: Unpublished data.
38. Kolouch, F., Jr.: The Lymphocyte in Acute Inflammation, Am. J. Path. **15**: 413, 1939.
39. Stedman, E.: The Chemistry of Cell Nuclei, Edinburgh M. J. **51**: 353, 1944.
40. Ehrich, W. E., and Harris, T. N.: The Site of Antibody Formation, Science **101**: 28, 1945.
41. Editorial: The Cellular Sources of Antibodies, J. A. M. A. **128**: 1232, 1945.

PRESERVATIVES FOR SYPHILITIC SERUM WITH SPECIAL REFERENCE TO THE USE OF MERTHIOLATE

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MANY serologists prefer to receive whole blood specimens for the performance of serodiagnostic tests for syphilis. Others request serum, when specimens must be shipped from a distant point, in order to eliminate hemolysis and decomposition of the blood, especially during summer temperatures. Contamination of such separated serum frequently occurs, as it is not always practicable to separate serum under sterile conditions, and unsatisfactory, anticomplementary, or less accurate serologic tests often result. Preservative substances may be added to the sera to inhibit bacterial growth, but these must not alter syphilitic reagin nor affect specificity. Preservatives have been employed in serum samples sent out for special serologic surveys[1] or for serodiagnostic evaluation studies, without general knowledge of specific data concerning the effects of preservation and of the aging of sera.

Merthiolate (sodium ethyl mercuri thiosalicylate)† was recommended[2] as a bacteriostatic agent in spinal fluid specimens in 1:1,000 to 1:10,000 concentrations, and it has been used by the authors for more than two years with notable success in spinal fluids sent by mail from a seven-state area. Crawford and Hertert[3] preserved sera for Wassermann tests by adding from 1.3 to 4.0 mg. per cubic centimeter of sulfanilamide, with no change in test reaction in eighteen days at room temperature and in observation of over 3,000 sera in duplicate. Perstein and Fishback[4] preserved Wassermann positive sera with 50 per cent glycerine, and when thus treated the reagin was found to survive prolonged heating at 56° centigrade. Rouslacroix and associates[5, 6] added 8-hydroxyquinoline sulfate (sunoxol) in 1:5,000 concentration to preserve whole blood for the Wassermann test.

A number of substances have been used as preservatives for immune sera and for biologic products. Marshall[7] reviewed and compared, from the standpoint of bacterial growth inhibition, some of the previously used serum preservatives. The following concentrations he found most favorable for bacteriostasis: tricresol, 0.2 per cent; 8-hydroxyquinoline sulfate (chinosol), 1:5,000; merthiolate, 1:5,000; glycerine, 1 per cent; and formalin, 0.2 per cent. Formalin had a destructive effect on serum antibodies, and the chinosol was not a good preservative. Boyd[8] found that toluene was not as satisfactory as the acriflavine or brilliant green dyes for preserving blood grouping sera. Jamieson and Powell[9] used merthiolate in vaccines, toxins, toxoids, bacteriophages, and sera and found minimum injury to labile antigen and antibody fractions. Its high

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†Throughout this article the term Merthiolate is used repeatedly and is so written, referring in each instance to this compound manufactured by Eli Lilly and Company, Indianapolis, Ind.

degree of solubility, freedom from precipitation and coagulation characteristics, as well as its germicidal effectiveness in protein solutions, allowed it to be particularly useful in materials of a labile nature affected by the usual preservatives. Morgan and co-workers[10] and Povitsky and Eisner[11] found merthiolate superior to phenol in the maintenance of the immunization properties of diphtheria toxoid. Waller[12] noted that merthiolate destroyed anti-Rh factors but not normal isoagglutinins in anti-Rh serum. Pressman and Grossberg[13] showed that it interfered with the specific precipitation of antisera against beef or sheep serum coupled with diazotized p-amino benzoic acid. Simmons and Gentzkow[14] noted that sodium fluoride may be used to preserve whole blood specimens sent by mail for chemical tests, the preservative effect being due to the inhibition of enzyme action.

The present study was undertaken to compare the preservative effect of those substances mentioned in the literature, with special reference to any interfering action on syphilitic reagin, and to include others which seemed worthy of trial. It was also planned to check more carefully the efficiency of the substances which gave the best preliminary results and to determine their desirability for routine use in sera mailed to a central laboratory for serologic testing.

EXPERIMENTAL

Comparison of Preservatives.—In addition to the preservative substances cited in the references, the following were used: sulfo-merthiolate (sodium p-ethyl mercuri thiosulfonate),* phenylmercuric borate, phenylmercuric nitrate, rivanol (ethoxydiamino-acridine),† zephiran chloride,† compound one (carboxymethoxylamine hemihydrochloride),‡[15] penicillin, chloroform, and thymol.

The preliminary tests were performed by adding nineteen different preservatives, in a presumably optimum range of concentrations, to 5 c.c. samples of a Seitz filtered pool of positive reacting syphilitic sera. The 5 c.c. samples of sera were distributed in 9 c.c. nonsterile vials with vinylite lined screw caps, as bacterial contamination was desired to test the maximum effectiveness of the preservatives. Including the control serum not containing a preservative, thirty-eight trials were set up in duplicate. The two groups were read by two serologists, and the tabular results are the average of their observations. When possible the preservative substances, diluted with physiologically normal saline, were added to the vials in 0.1 c.c. amounts, the original concentration of each being at such a level that upon the addition of the 5 c.c. serum sample the final desired dilution was attained; that is, 0.1 c.c. of 5.1 per cent merthiolate in 0.85 per cent saline was added to the vial for the 1:1,000 concentration. It was necessary to weigh out carefully into the test vials the phenylmercuric salts, rivanol, thymol, sodium fluoride, and sulfanilamide, because of their insolubility; and in these instances 0.1 c.c. of saline was added to the vials and to the control serum specimen for comparable effect. Phenol was dissolved in 95 per cent ethanol. Glycerine in 50 per cent concentration diluted the serum, so that quantitative Kahn test titers were multiplied by two to give comparable figures.

*Furnished by courtesy of Eli Lilly and Company, Indianapolis, Ind.
†Furnished by courtesy of Winthrop Chemical Company, Inc., New York, N. Y.
‡Furnished by courtesy of Dr. C. B. Favour, Peter Bent Brigham Hospital, Boston, Mass.

Standard and quantitative Kahn tests were performed on the serum pool at the beginning of the trials and on a 1 c.c. portion withdrawn from the prepared samples at intervals of 4, 7, 10, and 17 days. The preserved samples were examined macroscopically for contamination at each test interval, and stained smears of each serum were examined at the end of the seventeen days. An average of the duplicate test quantitative Kahn reactions, and of the relative degrees of contamination of the sera, is shown in Table I, together with concentra-

TABLE I. EFFECT OF TEST PRESERVATIVES IN VARYING CONCENTRATIONS ON POSITIVE SYPHILITIC SERUM, TWO RUNS AVERAGED (ORIGINAL KAHN REACTION, 80 KAHN UNITS)

SUBSTANCE	CONCENTRATION IN SERUM	QUANTITATIVE KAHN REACTIONS (KAHN UNITS)				CONTAMINATION				
						MACROSCOPIC				MICROSCOPIC
		4 DAYS	7 DAYS	10 DAYS	17 DAYS	4 DAYS	7 DAYS	10 DAYS	17 DAYS	17 DAYS
Control serum		80	40	40	20	2+	2+	2+	4+	4+
Merthiolate	1:1,000	80	80	80	40	±	±	±	±	–
Merthiolate	1:2,500	80	80	80	40	±	±	±	±	–
Merthiolate	1:5,000	80	80	80	40	±	1+	2+	1+	2+
Sulfo-merthiolate	1:1,000	80	40	40	40	±	±	±	±	±
Sulfo-merthiolate	1:2,500	80	80	80	40	±	±	±	±	–
Sulfo-merthiolate	1:5,000	80	80	40	40	1+	1+	2+	2+	3+
8-Hydroxyquinoline sulfate	1:1,500	80	80	80	40	1+	1+	1+	1+	±
8-Hydroxyquinoline sulfate	1:5,000	80	80	80	40	2+	2+	2+	2+	3+
8-Hydroxyquinoline sulfate	1:10,000	80	80	40	40	2+	2+	2+	2+	3+
Phenylmercuric borate	1:1,250	80	80	40	40	±	±	±	±	±
Phenylmercuric borate	1:5,000	80	80	80	80	±	±	±	±	±
Phenylmercuric borate	1:62,500	80	80	80	80	2+	2+	3+	4+	4+
Rivanol	1:10,000	80	80	120	40	2+	1+	2+	2+	4+
Rivanol	1:20,000	80	80	80	40	2+	2+	2+	2+	4+
Zephiran	1:25,000	80	80	40	40	2+	2+	2+	4+	3+
Zephiran	1:50,000	80	120	40	40	2+	1+	2+	4+	3+
Compound one	.25 mg./c.c.	80	80	80	80	2+	1+	2+	2+	4+
Penicillin	1 unit/c.c.	80	80	40	20	2+	1+	3+	4+	4+
Penicillin	2 units/c.c.	80	80	80	40	2+	1+	2+	4+	4+
Chloroform	.06 c.c.*/5 c.c.	40	40	40	40	±	±	±	±	–
Chloroform	.12 c.c./5 c.c.	4	4	20	40	2+	1+	1+	±	±
Thymol	10 mg./c.c.	0	0	0	0	Digested protein				–
Sodium fluoride	2 mg./c.c.	80	80	40	40	2+	2+	2+	2+	2+
Sodium fluoride	10 mg./c.c.	40	40	40	80	1+	1+	1+	±	–
Sulfanilamide	1.5 mg./c.c.	80	80	80	80	1+	1+	1+	1+	2+
Sulfanilamide	4 mg./c.c.	80	80	80	40	1+	±	±	±	±
Phenol	0.3%	40	40	40	40	±	1+	1+	2+	±
Phenol	0.5%	40	20	20	20	Insoluble precipitate				–
Formalin	0.2%	0	0	0	0	±	±	±	±	–
Tricresol	0.2%	40	40	40	20	±	3+	3+	3+	–
Glycerine	1%	80	80	80	80	2+	3+	3+	4+	3+
Glycerine	50%	80	80	80	80	–	–	–	–	–
Toluol	.06 c.c./5 c.c.	80	40	80	80	±	1+	1+	±	–
Acriflavine	1:100,000	80	80	40	40	2+	1+	2+	3+	4+
Acriflavine	1:200,000	80	40	40	40	2+	2+	2+	3+	4+
Brilliant green	1:3,000	80	80	40	40	1+	2+	1+	1+	±
Brilliant green	1:30,000	80	80	80	40	2+	2+	2+	2+	2+

*Note: 1 drop considered equal to .06 cubic centimeter.

tions of preservatives used. Because of marked drop in quantitative Kahn reactions, the following preservatives were eliminated from further consideration: chloroform, thymol, phenol, formalin, and tricresol. The following were eliminated because of failure to suppress bacterial contaminants in the concentrations employed: zephiran, penicillin, and acriflavine. Brilliant green imparted what we considered an undesirable color to the serum, and glycerine was deemed impractical for routine use because of its diluting effect, although it gave excellent preservation in 50 per cent concentration; therefore, these two substances were also eliminated.

TABLE II. EFFECT OF TEST PRESERVATIVES IN VARYING CONCENTRATIONS ON POSITIVE SYPHILITIC SERUM, TWO RUNS AVERAGED (ORIGINAL KAHN REACTION, 160 KAHN UNITS)

SUBSTANCE	CONCENTRATION IN SERUM	QUANTITATIVE KAHN REACTIONS (KAHN UNITS)				CONTAMINATION							
						MACROSCOPIC				CULTURAL			
		7 DAYS	10 DAYS	14 DAYS	21 DAYS	7 DAYS	10 DAYS	14 DAYS	21 DAYS	7 DAYS	10 DAYS	14 DAYS	21 DAYS
Control		120	80	80	20	2+	3+	4+	4+	4+	4+	4+	4+
Merthiolate	1:2,500	120	120	120	80	±	1+	2+	2+	2+	2+	2+	2+
Merthiolate	1:5,000	120	120	120	80	±	1+	2+	2+	2+	2+	2+	2+
Sulfo-merthiolate	1:2,500	120	80	80	80	±	1+	2+	2+	2+	2+	2+	2+
Sulfo-merthiolate	1:5,000	120	120	120	120	±	±	1+	2+	1+	2+	2+	2+
8-Hydroxyquinoline sulfate	1:1,000	120	120	120	80	±	±	±	1+	-	-	±	±
8-Hydroxyquinoline sulfate	1:2,000	160	120	160	80	-	-	±	1+	-	-	1+	±
Phenylmercuric borate	1:5,000	120	120	120	80	±	±	1+	1+	-	±	1+	±
Phenylmercuric nitrate	1:5,000	120	120	120	80	±	1+	1+	3+	-	1+	2+	±
Phenylmercuric nitrate	1:50,000	160	120	120	80	1+	1+	2+	2+	1+	2+	2+	3+
Rivanol	1:5,000	120	80	40	40	2+	3+	3+	2+	2+	2+	3+	3+
Compound one	2 mg./c.c.	160	80	120	120	2+	3+	1+	2+	2+	2+	2+	2+
Compound one	1 mg./c.c.	160	80	80	80	2+	1+	1+	2+	2+	2+	2+	2+
Sodium fluoride	5 mg./c.c.	160	160	120	120	±	±	1+	1+	-	±	1+	1+
Sodium fluoride	7.5 mg./c.c.	160	160	160	120	±	-	±	1+	-	-	±	±
Sodium fluoride	10 mg./c.c.	160	160	160	160	±	-	±	±	-	-	±	±
Sulfanilamide	4 mg./c.c.	160	160	120	80	2+	1+	2+	1+	2+	1+	1+	1+
Sulfanilamide	5 mg./c.c.	160	120	120	120	2+	1+	2+	2+	2+	1+	1+	1+
Toluol	.06 c.c.*/5 c.c.	160	120	80	120	±	±	-	±	-	-	-	±
Toluol	.12 c.c./5 c.c.	120	120	80	120	±	-	-	±	-	±	-	-

*Note: 1 drop considered equal to .06 cubic centimeter.

Ten preservatives were compared in positive serum for trial two, the range of dilutions making a total of twenty different tests in duplicate. Test intervals were at 7, 10, 14, and 21 days. In addition to observing macroscopic contamination, 0.1 c.c. of each serum was inoculated into 5 c.c. of nutrient broth at each test interval, and the relative degree of developing growth was read after forty-eight hours' incubation. Contamination and the quantitative Kahn reactions are shown in Table II. All substances showed some promise of a preservative effect, with the exception of rivanol, although certain dilutions of the substances were relatively ineffective, for example, phenylmercuric nitrate, 1:50,000.

The following six preservatives were compared with a control serum in trial three: merthiolate, 1:1,000 and 1:2,500; sulfo-merthiolate, 1:2,500; 8-hydroxyquinoline sulfate, 1:2,000; sodium fluoride, 10 mg. per cubic centimeter, 12.5 mg. per cubic centimeter, and 15 mg. per cubic centimeter; sulfanilamide, 5 mg. per cubic centimeter; and toluol, 1 drop (0.06 c.c. per 5 c.c.). Three series were set up, with positive reacting serum in sterile and nonsterile vials and with negative serum in nonsterile vials. Aseptic precautions were used with the "sterile" series. The results obtained are not tabulated, for all sera, including the controls, maintained 40 Kahn units for three weeks with the exception of the 1:1,000 merthiolate serum, which dropped to 20 units in two weeks for the nonsterile vials and to 20 units in three weeks for the sterile vials. The positive serum control showed 1 plus and 2 plus contamination in both series throughout. The 8-hydroxyquinoline sulfate serum showed plus-minus contamination in two weeks, and sulfanilamide serum plus-minus to 1 plus contamination in one to two weeks, and that with toluol allowed plus-minus to 1 plus contamination throughout. It was probable that the Seitz filtration did not completely sterilize the positive serum pool. In the negative serum trials, in nonsterile vials, the control serum and toluol-preserved serum showed 1 plus contamination. A nonspecific precipitate developed after two weeks in the 8-hydroxyquinoline sulfate serum.

At this point, sodium fluoride appeared advantageous. Vials were prepared by adding the required amount of 1:25 aqueous solution of the salt and drying in a warm temperature, so that concentrations of 5 mg. per cubic centimeter and 7.5 mg. per cubic centimeter in serum would be attained by adding 3, 4, or 5 c.c. of serum to the proper vials. Seventeen vials each of 5 mg. per cubic centimeter and 7.5 mg. per cubic centimeter concentration were tested with positive and negative sera. Sodium fluoride was shown to affect the specificity of the Kahn test in one week's time in 8 of 11 negative sera in 5 mg. per cubic centimeter vials and in all 14 of 7.5 mg. per cubic centimeter negative serum vials. Kahn reactions as high as 1 3 4 were obtained in the standard test.

In Table III is shown the effect of merthiolate in concentrations from 1:1,000 to 1:10,000, compound one at 1 mg. per cubic centimeter and sulfanilamide at 5

TABLE III. EFFECT OF TEST PRESERVATIVES IN VARYING CONCENTRATIONS ON POSITIVE SYPHILITIC SERUM, TWO RUNS AVERAGED (ORIGINAL KAHN REACTION, 40 KAHN UNITS)

SUBSTANCE	CONCENTRATION IN SERUM	QUANTITATIVE KAHN REACTIONS (KAHN UNITS)				CONTAMINATION							
						MACROSCOPIC				CULTURAL			
		1 WEEK	2 WEEKS	3 WEEKS	4 WEEKS	1 WEEK	2 WEEKS	3 WEEKS	4 WEEKS	1 WEEK	2 WEEKS	3 WEEKS	4 WEEKS
Control		40	40	40	20	1+	1+	3+	4+	1+	4+	3+	3+
Merthiolate	1:1,000	40	40	40	40	–	–	–	–	–	–	–	–
Merthiolate	1:2,500	40	40	40	40	–	–	–	–	–	–	–	–
Merthiolate	1:5,000	40	40	40	40	–	–	–	–	–	–	–	–
Merthiolate	1:7,500	40	40	40	40	–	–	–	–	–	–	–	–
Merthiolate	1:10,000	80	40	40	40	–	–	–	–	–	–	–	–
Compound one	1 mg./c.c.	40	40	40	20	–	1+	3+	4+	±	2+	3+	3+
Sulfanilamide	5 mg./c.c.	40	40	40	40	–	±	1+	2+	±	1+	1+	1+

mg. per cubic centimeter on positive Kahn serum giving a titer of 40 Kahn units. Only the control serum and that containing compound one decreased to 20 Kahn units in four weeks' time. The control serum, that containing compound one, and that with sulfanilamide showed contamination.

Further comparisons were made of the effectiveness of merthiolate in 1:1,000, 1:2,500, and 1:5,000 concentrations, compound one at 2 mg. per cubic centimeter of serum, 8-hydroxyquinoline sulfate at 1:2,000, phenylmercuric nitrate and borate at 1:5,000, sulfanilamide at 5 mg. per cubic centimeter, and toluol at 1 drop per 5 cubic centimeter. Sulfo-merthiolate was not tested further, since it showed no advantages over merthiolate. A slightly different method of vial preparation was used for these comparisons, in that the preservatives were dissolved in water, except for the phenylmercuric salts which were made up 1:800 in 95 per cent ethanol (not completely soluble). The sulfanilamide was used in a 2 per cent solution in 95 per cent ethanol, and the toluol was added to the serum in liquid form. The sterile vials containing the preservatives, with the tops loosened, were placed in a desiccator until the diluent had evaporated.

TABLE IV. EFFECT OF TEST PRESERVATIVES IN VARYING CONCENTRATIONS ON POSITIVE SYPHILITIC SERUM, TWO RUNS AVERAGED (ORIGINAL KAHN REACTION, 40 KAHN UNITS)

SUBSTANCE	CONCENTRATION IN SERUM	QUANTITATIVE KAHN REACTIONS (KAHN UNITS)				CONTAMINATION							
						MACROSCOPIC				CULTURAL			
		1 WEEK	2 WEEKS	3 WEEKS	4 WEEKS	1 WEEK	2 WEEKS	3 WEEKS	4 WEEKS	1 WEEK	2 WEEKS	3 WEEKS	4 WEEKS
Control		40	20	4	4	4+	4+	4+	4+	4+	3+	3+	3+
Merthiolate	1:1,000	40	40	40	40	–	–	–	–	–	–	–	–
Merthiolate	1:2,500	40	40	40	40	–	–	–	–	–	–	–	–
Merthiolate	1:5,000	40	40	40	40	–	–	–	–	–	–	–	–
Compound one	2 mg./c.c.	40	40	40	20	–	–	–	±	–	–	–	±
8-Hydroxyquinoline sulfate	1:2,000	40	40	40	40	±	Precipitate			–	–	–	–
Phenylmercuric borate	1:5,000	40	40	40	40	–	±	1+	2+	–	±	1+	1+
Phenylmercuric nitrate	1:5,000	40	40	40	40	1+	1+	1+	2+	±	±	±	1+
Sulfanilamide	5 mg./c.c.	80	40	40	40	–	±	±	±	–	1+	1+	1+
Toluol	.06 c.c./5 c.c.	80	40	40	20	2+	1+	2+	2+	1+	2+	2+	2+

Five cubic centimeters of the test sera were added to each vial and were mixed thoroughly. Two duplicate groups of positive sera (one of 40 and one of 20 units) were tested, as were three of negative sera. Tabulation of all results is not shown. In the positive serum of 20 units, all preservatives maintained the original 20 Kahn unit titer for four weeks, with only toluol allowing slight contamination. In the positive serum of 40 units, which was set up with a Seitz filtered serum that had previously shown gross contamination, phenylmercuric borate, nitrate, sulfanilamide, and toluol did not restrain bacterial growth; whereas, merthiolate completely inhibited growth (Table IV). Compound one and toluol allowed a quantitative Kahn decrease in titer from 40 units to 20 units in four weeks.

TABLE V. EFFECT OF MERTHIOLATE 1:2,500 ON KAHN REACTIONS OF SERA WHICH STOOD ONE, TWO, AND THREE WEEKS AT ROOM TEMPERATURE

ORIGINAL KAHN TEST REACTION GROUP*	CHANGE FROM ORIGINAL KAHN REACTIONS	ONE WEEK		TWO WEEKS		THREE WEEKS	
		NUMBER OF SERA	PER CENT	NUMBER OF SERA	PER CENT	NUMBER OF SERA	PER CENT
Positive (4 Kahn units or above)	No change†	127	63.5	112	56.0	88	48.9
	Slight change‡	12	6.0	22	11.0	29(1‖)	16.1
	Significant change§	6	3.0	11(2‖)	5.5	10(2‖)	5.6
Positive (2 plus and 3 plus reactions)	No change†	28	14.0	29	14.5	26	14.4
	Slight change‡	7(5¶)	3.5	5(3¶)	2.5	7(2¶)	3.9
	Significant change§	1‖	0.5	2(1‖)	1.0	3(‖)	1.7
Doubtful (1 plus reaction)	No change†	14	7.0	10	5.0	8	4.4
	Slight change‡	5(4¶)	2.5	9(7¶)	4.5	9(8¶)	5.0
Totals		200	100.0	200	100.0	180	100.0

Note: 23 negative sera were also included in this trial for the two weeks period and 19 for the three-week period. Four gave slight plus-minus or 1 plus reactions in single tubes on rare occasions but never sufficient to change the report to doubtful.

*Based on one reading, a 3-tube total of 5 to 12 plus is Positive; 2 to 4 plus, Doubtful; 0 to 1 plus, Negative.

†Reaction remained in same group, or quantitative test end point differed in not more than one dilution.

‡Positive to doubtful, doubtful to negative, or a quantitative end point difference of not more than three dilutions.

§Positive to negative or a quantitative end point difference of four dilutions, or more.

‖Contaminated.

¶No greater difference than 3 plus but sufficient to change the reported reaction.

In the three trials with negative sera, totaling sixty tests, the substances listed in Table IV did not affect the specificity of the Kahn test over a four-week period, although 8-hydroxyquinoline sulfate gave a confusing extraneous precipitate in two weeks in all instances. In two of these series, toluol allowed contamination to develop.

Practical Use of Merthiolate.—The preceding experiments demonstrated an apparent superiority of merthiolate; hence, it was chosen for further trial. Screw

TABLE VI. EFFECT OF MERTHIOLATE 1:1,000 ON KAHN REACTIONS OF SERA WHICH STOOD ONE, TWO, AND THREE WEEKS AT ROOM TEMPERATURE

ORIGINAL KAHN TEST REACTION GROUP*	CHANGE FROM ORIGINAL KAHN REACTIONS	ONE WEEK		TWO WEEKS		THREE WEEKS	
		NUMBER OF SERA	PER CENT	NUMBER OF SERA	PER CENT	NUMBER OF SERA	PER CENT
Positive (4 Kahn units or above)	No change†	39	48.1	33	40.7	29	36.7
	Slight change‡	19	23.5	19	23.5	22	27.9
	Significant change§	3	3.7	9	11.1	9(‖)	11.4
Positive (2 plus and 3 plus reactions)	No change†	9	11.1	8	9.9	5(‖)	6.3
	Slight change‡	4(¶)	4.9	1¶	1.2	1	1.3
	Significant change§	2	2.5	6	7.4	8	10.1
Doubtful (1 plus reaction)	No change†	2	2.5	2	2.5	2	2.5
	Slight change‡	3(2¶)	3.7	3(2¶)	3.7	3(2¶)	3.8
Totals		81	100.0	81	100.0	79	100.0

Note: 12 negative sera were also included in this trial for the three-week period, one of which gave a false doubtful reaction in two weeks (±11) but a negative in three weeks (−−1).

*Based on one reading, a 3-tube total of 5 to 12 plus is Positive; 2 to 4 plus, Doubtful; 0 to 1 plus, Negative.

†Reaction remained in same group, or quantitative test end point differed in not more than one dilution.

‡Positive to doubtful, doubtful to negative, or a quantitative end point difference of not more than three dilutions.

§Positive to negative, or a quantitative end point difference of four dilutions, or more.

‖Contaminated.

¶No greater difference than 3 plus, but sufficient to change the reported reaction.

cap vials of 9 c.c. capacity were autoclaved. Two series were so prepared that upon the addition of 3, 4, or 5 c.c. of serum the resultant concentrations of 1:2,500 and 1:1,000 were obtained. To accomplish this, six tests of these sterile vials were prepared containing, respectively, 0.1 c.c. of merthiolate prepared in 1:33.3, 1:25, 1:20, 1:83.3, 1:62.5, and 1:50 aqueous dilutions. Addition of 3, 4, or 5 c.c. of serum, respectively, to vials containing the first three concentrations

TABLE VII. SERA SHOWING SIGNIFICANT CHANGES IN KAHN TITER

SPECIMEN	ORIGINAL REACTION	ONE WEEK	TWO WEEKS	THREE WEEKS	CLINICAL NOTES
			1:2,500 Merthiolate		
3	-23	--4	--2	---	Early latent syphilis; previous R
6	360 K.U.	200 K.U.	120 K.U.	120 K.U.	Neurosyphilis, asymptomatic; previous R
18	360 K.U.	360 K.U.	200 K.U.	200 K.U.	Gonorrhea; early latent syphilis; R at time of specimen
20	400 K.U.	240 K.U.	160 K.U.	160 K.U.	No record
26	344	---	---	-11	No record
55	±44	-22	--±	---	Gonorrhea; R at time of specimen; incomplete history
64	360 K.U.	200 K.U.*	200 K.U.*	200 K.U.*	Primary syphilis; R at time of specimen
67	160 K.U.	80 K.U.	---	---	Gonorrhea; primary syphilis; R at time of specimen
74	4 K.U.	244	-±±*	---*	Primary syphilis, untreated
81†	4 K.U.	---	---	---	False positive, incorrectly recorded as early latent; inductee, no R; titer fluctuated, 360 K.U. to negative within 20 days
92	-34	---*	---*	---*	Latent syphilis, previous R; gonorrhea, R now
117	4 K.U.	4 K.U.	---*	---*	Early latent, completed second R 2 days before
148	20 K.U.	-44	-±1	--1	Early latent, no R; quantitative Kahn fluctuated, 4 to 40 K.U.
150†	4 K.U.	---	---	---	Same as Specimen 81
			1:1,000 Merthiolate		
12	±24	-13	-12	--±	Late latent syphilis
26	344	---	---	---	No record
32	-44	112	---	--±	Late latent syphilis
55	±44	---	---	---	Same as Specimen 55 above
63‡	4 K.U.	-±3	---	---	False positive due to smallpox vaccination month before
72‡	4 K.U.	-11	---	---	Same as Specimen 63
81†	4 K.U.	---	---	---	Same as Specimen 81 above
83	200 K.U.	120 K.U.	20 K.U.	20 K.U.	Early latent syphilis, no R
92	-34	---	---	---	Same as Specimen 92 above
94	344	--1	--±	---	Questionable primary; observed one month, dismissed without R
132	4 K.U.	-23	---	---	No record
136	-44	-±4	---	---	No record
142	-44	-23	-23	---	No record
148	200 K.U.	-44	--1	--1*	Same as Specimen 148 above
149	-44	-24	---	--1	Mucocutaneous relapse
150†	4 K.U.	---	---	---	Same as Specimen 81 above
152	-44	-±4	---	---	Old syphilis, previous R; chancroid or lymphogranuloma at time of specimen, no R

K.U., Kahn units.
R, Treatment.
*Contamination.
†Same patient.
‡Same patient.

gives a 1:1,000 concentration. Similarly, such quantities in the last three tubes gives a 1:2,500 concentration.

The blood samples were obtained in 30 c.c. vacuum venules from patients at the local venereal disease clinic. They were refrigerated until the sera were separated and placed in the vials containing merthiolate. In some instances, serum stood on the clot for as long as three days. The sera were then kept at room temperature in the dark, and 1 c.c. portions were removed immediately after adding the serum and at intervals of one, two, and three weeks, unless they were sooner exhausted. These portions were centrifuged, decanted, inactivated for thirty minutes at 56° C., and then tested by the Kahn standard and by the quantitative technique when indicated.

Results with the two series are shown in Tables V and VI. The original Kahn reactions were separated into a quantitative group showing 4 Kahn units or more, a positive group showing 2 or 3 plus reactions, a doubtful group, and a negative group. Changes at one, two, and three weeks were tabulated as showing "no change," "slight change," or "significant change" (see footnote criteria in Tables V and VI. The 1:1,000 merthiolate gave a greater percentage of significant changes than did the 1:2,500 concentration, and both series showed greater decreases in titer after two and three weeks than at one week. One negative serum (1:1,000 preservation) became doubtful (± 11) in two weeks but at the end of three weeks reverted to negative (– – 1). Otherwise there were no changes in specificity.

TABLE VIII. DIFFERENCES IN KAHN REACTIONS OVER TWO- OR THREE-WEEK PERIODS WITH SAME SERA AT TWO CONCENTRATIONS OF MERTHIOLATE (1:2,500 AND 1:1,000)

DIFFERENCE IN REACTIONS BETWEEN THE TWO	NUMBER OF SERA	NUMBER GIVING HIGHER TITER AT 1:2,500	NUMBER GIVING HIGHER TITER AT 1:1,000
Agreement	63	–	–
Slight disagreement	19	15	4
Significant disagreement	11	10	1
Totals	93	25	5

After three weeks, of the sera in the 1:1,000 series, 45.5 per cent showed "no change" in titer, 33 per cent showed a "slight change," and 21.5 per cent showed a "significant change." The sera in the 1:2,500 series gave the following at the end of three weeks: 67.7 per cent "no change"; 25 per cent "slight change"; 7.3 per cent "significant change." In Table VII are listed the cases with significant changes in the Kahn reaction, and notes as available are included.

It was possible to preserve ninety-three specimens in both concentrations. The results are summarized in Table VIII. The 1:2,500 dilution gave better results in a proportion of 5 to 1. In Table IX are listed the detailed results in those cases of significant disagreement.

Of 223 sera preserved by the 1:2,500 dilution, 14 showed contamination: 5 at one week, 7 at two weeks, and 2 at the end of the third week. Of 93 sera preserved by the 1:1,000 dilution only two specimens were contaminated.

TABLE IX. TABULATION OF DUPLICATE SERA SHOWING SIGNIFICANT DISAGREEMENT WHEN PRESERVED (1:2,500 AND 1:1,000 WITH MERTHIOLATE)

SPECIMEN	ORIGINAL REACTIONS		ONE WEEK		TWO WEEKS		THREE WEEKS	
	1:2,500	1:1,000	1:2,500	1:1,000	1:2,500	1:1,000	1:2,500	1:1,000
32	-44	-44	134	112	123*	---	-34*	--±
39	20 K.U.	40 K.U.	40 K.U.	-24	40 K.U.	-12	40 K.U.	No test
63	4 K.U.	4 K.U.	-34	-±3	-24	---	--2	---
72	4 K.U.	4 K.U.	-44*	-11	-23*	---	-±3*	---
83	200 K.U.	200 K.U.	200 K.U.	120 K.U.	200 K.U.	20 K.U.	120 K.U.	20 K.U.
94	344	344	-44	--1	-44	--±	-34	---
123	---	---	---	-±±	---	±11	---	--1
132	4 K.U.	4 K.U.	-44	-23	-24	---	-44	---
136	-44	-44	-24	-±4	-23	---	-44	---
149	-44	-44	-24	-24	-44	---	-24	--1
152	-44	-44	-44	-±4	-44	---	-44	---

K.U., Kahn units.
*Serum showed contamination.

DISCUSSION

Inaccurate or unsatisfactory Kahn reactions have undoubtedly resulted, in the past, from either distant shipment of plain sera or the use of various preservatives unknown to the serologist. It is possible that syphilitic serum loses reagin to some extent after it has been separated from the clot,[16] and unless shipped as whole blood, or with a satisfactory preservative, undersensitive tests will be reported from the distant laboratory.

The present study indicates that none of the preservatives, in concentrations tested, shows a better preservative effect on syphilitic serum than merthiolate. Sulfo-merthiolate appeared to be almost as satisfactory. Compound one in a concentration of 2 mg. per cubic centimeter was useful in this application. Neither sulfanilamide, phenylmercuric borate or nitrate, nor toluol gave consistent bacterial inhibition in sera, although reagin content did not decrease appreciably in their presence. Crawford and Hertert's[3] recommendation of sulfanilamide was not confirmed in our limited study. The phenylmercuric salts are impractical because of their low solubility. Glycerine, which in 50 per cent concentration preserved reagin, cannot be used routinely because of the high dilution required.

The use of sterile rather than nonsterile vials in the first part of the study did not prove advantageous, since bacterial contamination was desired to test the effectiveness of the preservatives. Either the vials or the serum furnished the contaminants in most instances, as would often occur in practical application of preservatives to serum.

Merthiolate was effective bacteriostatically in 1:1,000 to 1:10,000 concentrations. The 1:1,000 concentration gave a greater percentage of decreased Kahn titers and one nonspecific reaction; therefore, 1:2,500 is recommended for practical usage. Merthiolate 1:2,500 did not markedly interfere with reagin over a three-week period in 92.7 per cent of 180 positive sera. The drop in one week was less, 96.5 per cent of 200 sera remaining unchanged or dropping insignificantly. The comparative figure at one week's time for the 1:1,000 series was 93.8 per cent of 81 sera. Under present conditions of transport, sera should reach a laboratory within one week.

The seven cases (3.5 per cent of total) in the 1:2,500 series, which changed significantly in one week (see Table VII), would have given one false negative (Specimen 92), and it was contaminated. Three others (Specimens 26, 81, and 150) were from patients not known to have syphilis. The other three (Specimens 6, 20, and 64), though reduced in reagin, still gave strong positives. Of twenty-six cases showing significant reduction of reagin in Table VII, for which clinical data were available, nine were either receiving penicillin or had just completed such treatment at the time the blood was obtained. Undue significance must not be attributed to this, for many of the other cases in this study were likewise being treated or had just completed treatment, yet showed no decrease in reagin.

False positive sera may decrease in titer. Three specimens were obtained from a patient (Case 1) diagnosed as having false positive reactions due to recent immunizations and smallpox vaccination, also having a history of upper respiratory infection. Another patient (Case 2) had a false positive reaction due to a malaria attack two and one-half weeks previously. A third patient (Case 3), a 17-year-old inductee, incorrectly recorded as having early latent syphilis, denied exposure and had other quantitative fluctuations from 360 Kahn units to negative. These reactions are shown in Table X.

TABLE X. KAHN REACTIONS SHOWING DROPS IN TITER ASSOCIATED WITH FALSE POSITIVE REACTIONS FROM THREE PATIENTS

DATE	CASE	SPECIMEN	ORIGINAL		ONE WEEK		TWO WEEKS		THREE WEEKS	
			1:2,500	1:1,000	1:2,500	1:1,000	1:2,500	1:1,000	1:2,500	1:1,000
1/31	1	58	20 K.U.	20 K.U.	444	244	-23	-14	-23	-23
2/5	1	63	4 K.U.	4 K.U.	-34	-±3	-24	---	--2	---
2/7	1	72	4 K.U.	4 K.U.	-44	-11	-23	---	-±3	---
12/21	2	16	±12	-12	-11	---	-±±	---	---	---
2/12	3	81	4 K.U.	4 K.U.	---	---	---	---	---	---
2/27	3	150	4 K.U.	4 K.U.	---	---	---	---	---	---

K.U., Kahn units.

Five sera from both series showed very slight increases in titer after two weeks, one of which resulted in a nonspecific Kahn reaction (– 11) in 1:1,000 merthiolate concentration, the only instance of the development of a false reaction with merthiolate.

Contamination either enhanced or decreased Kahn titers in sixteen instances. Given sufficient inoculum, 1:2,500 merthiolate will allow contaminants to increase, usually within two weeks. Tests on such sera should be carefully considered and rechecked with another specimen if possible.

The criteria for designating change in reaction as "no change," "slight change," or "significant change" were devised so that they would coincide with a physician's usual interpretation of such reports were he to receive them in following a case. A false drop in quantitative titer of three dilutions in a preserved sample might be misleading. Quantitative titers on preserved sera should be interpreted with care.

SUMMARY AND CONCLUSIONS

A study of the preservation of syphilitic reagin as demonstrated by the Kahn test is presented.

Twenty preservative substances were compared for their effect on positive syphilitic sera and on negative sera over periods of two and one-half to four weeks at room temperature. None gave greater preservation than merthiolate (sodium ethyl mercuri thiosalicylate), which was effective, depending on the amount of original contamination, in concentrations of 1:1,000 to 1:10,000. A comparison of two concentrations revealed that serum containing 1:2,500 merthiolate showed less drop in reagin content than that with the 1:1,000 amount. Test specificity was not affected when concentrations less than 1:1,000 were used.

A practical method of preserving sera with merthiolate 1:2,500 for shipment by mail is described. Less than 4 per cent of 200 sera so tested showed a significant loss in titer in one week's time, and only 7.3 per cent showed a significant decrease in three weeks. The use of such a method of preservation would result in more accurate serology in instances where whole blood cannot be sent to a laboratory without danger of gross deterioration. The method is not intended to supplant shipments of whole blood when such can be made satisfactorily.

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REFERENCES

1. Scott, V., Rein, C. R., Schamberg, I. L., Moore, J. E., and Eagle, H.: The Serologic Differentiation of Syphilitic and False Positive Sera, Am. J. Syph., Gonor. & Ven. Dis. **29**: 505-528, 1945.
2. Harris, A., and Mahoney, J. F.: Merthiolate as an Effective Bacteriostatic Agent in Spinal Fluid Specimens, Ven. Dis. Inform. **25**: 46, 1944.
3. Crawford, J. P., and Hertert, L. D.: Preservation of Wassermann Sera by Means of Sulfanilamide, Mil. Surgeon **93**: 274-275, 1943.
4. Perstein, F., and Fishback, H. R.: Glycerine Preservation of Serologic Test Materials, and the Influence of Heat on Glycerinated Wassermann-Positive Sera, J. Lab. & Clin. Med. **14**: 969-973, 1929.
5. Rouslacroix, A., Boyer, L., and Gastinel, R.: Action de quelques antiseptiques sur les éléments et les processus qui conditionnent la réaction de Bordet-Wassermann, Compt. rend. Soc. de biol. **125**: 71-74, 1937.
6. Rouslacroix, A., Boyer, L., and Gastinel, R.: Utilisation du pouvoir antiseptique du sulfate neutre d'orthoxyquinoléine pour la conservation du sang destiné à la réaction de Bordet-Wassermann, Compt. rend. Soc. de biol. **125**: 74-76, 1937.
7. Marshall, M. S.: Serum Preservatives, California & West. Med. **41**: 94-98, 1934.
8. Boyd, W. C.: Production and Preservation of Specific Anti-Sera for Blood Group Factors, A, B, M, and N, J. Immunol. **37**: 65-75, 1939.
9. Jamieson, W. A., and Powell, H. M.: Merthiolate as a Preservative for Biologic Products, Am. J. Hyg. **14**: 218-224, 1931.
10. Morgan, L. C., Jamieson, W. A., and Powell, H. M.: Merthiolate as a Preservative for Biologic Products. II. The Production and Preservation of Diphtheria Toxoid, J. Immunol. **25**: 121-126, 1933.
11. Povitzky, O. R., and Eisner, M.: Merthiolate Versus Phenol as a Preservative for Diphtheria Toxoids—Diluted and Undiluted at Icebox and Room Temperatures, J. Immunol. **28**: 209-213, 1935.
12. Waller, R. K.: The Action of Sodium Ethylmercurithiosalicylate on Human Anti-Rh Serums, Am. J. Clin. Path., Tech. Sect. **8**: 116-117, 1944.
13. Pressman, D., and Grossberg, A. L.: Effect of Merthiolate (Lilly) on Certain Specific Precipitation Reactions, Science **101**: 254-255, 1945.
14. Simmons, J. S., and Gentzkow, C. J.: Laboratory Methods of the United States Army, Philadelphia, 1944, Lea and Febiger, p. 198.
15. Favour, C. B.: Personal communication.
16. Barnard, R. D., and Rein, C. R.: Personal communications.

SECONDARY BACILLUS PYOCYANEUS INFECTION IN MENINGITIS FOLLOWING INTRATHECAL PENICILLIN THERAPY

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FROM time to time there have been reported in the literature cases of secondary meningeal infections caused by intrathecal punctures. In 1911, Schlagenhaufer[1] reported five cases of *Bacillus pyocyaneus* meningitis resulting from contaminated saline used in mixing spinal anesthetics administered to these patients. A total of eleven cases of *B. pyocyaneus* meningitis after diagnostic lumbar punctures has been reported by Sonnenschein,[2] Levy and Cohen,[3] Evans,[4] and Wise and Musser.[5] Kerman and associates,[6] in 1943, reported a case of *B. pyocyaneus* meningitis following pneumoencephalogram. In this instance a wash bottle through which helium was passed was found to be the source of the contamination. In a review of the literature Kerman stated that among the fifty-two cases reported there was a mortality rate of 58 per cent, despite various types of therapy, including the sulfonamides.

During 1945, one of us (E. A.) observed four patients with pneumococcus meningitis who were treated with penicillin, intramuscularly and intrathecally, and subsequently developed a secondary *B. pyocyaneus* meningitis. Two of the patients were at the Willard Parker Hospital, where bacteriologic investigation established the source of the secondary infection.

CASE REPORTS

CASE 1.—Patient A. M., a 9-month-old Puerto Rican female, was admitted Dec. 16, 1944, with a diagnosis of pertussis because of a history of coughing and vomiting for six days. When first seen the patient had convulsions, the anterior fontanel was bulging, and dilated veins were prominent over the scalp. The temperature was 104° F.; pulse, 160; and respirations, 52. A spinal tap was done, and 10 c.c. of cloudy fluid were removed under increased pressure. On direct examination this fluid showed the presence of pneumococcus type XVIII.

Treatment with 10,000 units of penicillin intramuscularly every three hours and daily intrathecal injections of 10,000 units of penicillin in 10 c.c. of saline was started. Orally, 5 Gm. of sulfadiazine were given daily to maintain blood levels between 9 to 14 mg. per cent. After twenty-two days, because the spinal fluid cultures continued to show pneumococci, the dosage of the intrathecal penicillin was increased to 20,000 units in bi-daily injections and continued for twelve days.

There were many exacerbations in the course of this patient's illness, but as the temperature was normal from Jan. 10 to 19, 1945 (Fig. 1), intrathecal treatment was discontinued on the nineteenth. On January 22 the temperature rose, the patient again became irritable, and the anterior fontanel became tense. Pneumococcus type XVIII was again recovered from the spinal fluid, and the bi-daily intrathecal injections of penicillin were

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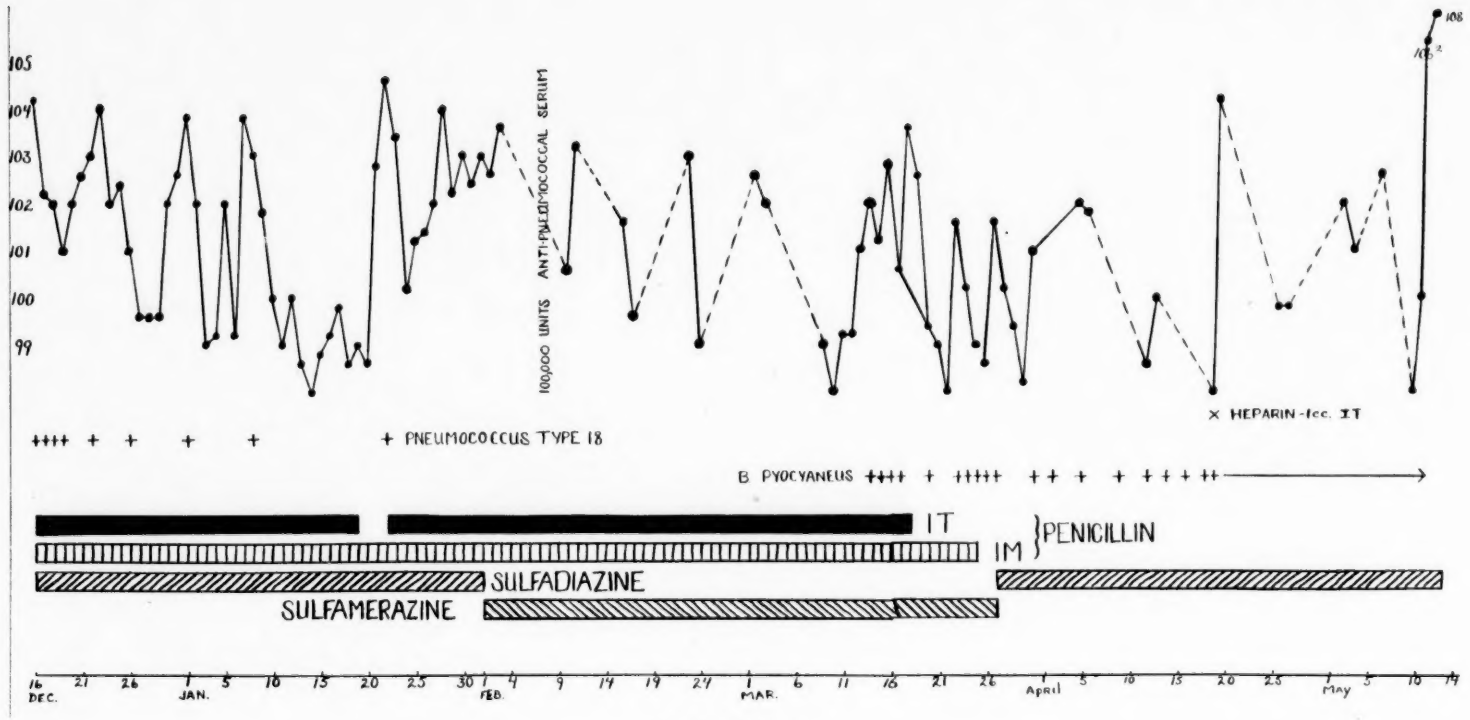
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Fig. 1.—Case 1.

resumed. For the next three weeks the temperature ranged from 103 to 99° F., and signs of meningeal irritation persisted, even though cultures of the spinal fluid showed no growth after January 22. On February 8, 100,000 units of type XVIII antipneumococcus serum were given intravenously. The temperature elevation gradually decreased and was normal from March 7 to 13. During this period spinal fluid drainage was carried out, and intrathecal penicillin was administered every morning and in the evenings only when the fontanel was bulging.

On the evening of March 13, 1945, the patient vomited, and at 2 A.M. on March 14 the temperature rose to 102° Fahrenheit. The fontanel was bulging, eyes protruded, and the patient was irritable. Very thick purulent spinal fluid was obtained under increased pressure, and 10,000 units of penicillin were instilled after drainage. This treatment was continued until March 17 when a report was obtained that a gram-negative bacillus had been cultured from all specimens of spinal fluid from March 14. The bacillus was identified later as *B. pyocyaneus*. From March 18 to the day of death no intrathecal penicillin was administered, but bi-daily lumbar punctures were performed to relieve pressure and to drain off fluid. One cubic centimeter of heparin was given intrathecally to prevent adhesions. Intramuscular injections of penicillin were not given after March 25. A total of 1,580,000 units of penicillin intrathecally and 7,430,000 units intramuscularly were administered. No local abscesses were ever noted in the buttocks in the region of intramuscular injections.

The spinal fluid cultures were persistently positive for *B. pyocyaneus*, and the patient grew steadily worse and died on May 14, 1945.

CASE 2.—Patient, B. H., an 83-year-old white woman, was admitted Feb. 24, 1945, to another hospital, with the history of pain in the right ear for two days. A spinal tap was done and 20,000 units of penicillin intrathecally and 20,000 units intramuscularly were given. The patient was transferred to Willard Parker Hospital on the same day.

On admission she was unconscious and had a stiff neck, positive Kernig sign, and diminished deep reflexes. She was given an infusion of 5 per cent glucose in saline and sodium sulfadiazine intravenously. On February 27 it was reported that the spinal fluid and blood cultures showed pneumococcus type XIV, and treatment with 20,000 units of penicillin once daily intrathecally and 15,000 units intramuscularly every three hours was instituted. Sulfadiazine therapy was also continued.

The patient became rational on the third hospital day; the temperature gradually fell and reached normal on Barch 11, 1945. On March 14 at 2 A.M. the temperature again rose to 103.4° F. (Fig. 2), and the patient again became slightly irrational. There was marked stiffness of the neck and positive Kernig sign. At this time purulent spinal fluid was obtained under increased pressure.

On March 17 the laboratory reported that a gram-negative bacillus was cultured from all spinal fluid specimens since March 14 and that this microorganism was also a *B. pyocyaneus*. Drainage of the spinal fluid was done thereafter without injection of penicillin. The patient continued to receive sulfadiazine and intramuscular injections of penicillin. The temperature rose from 103 to 105° fahrenheit. She became comatose, developed râles at the lung bases and scattered areas of bronchial breathing. She died on March 28, 1945. This patient received a total of 380,000 units of penicillin intrathecally and 2,680,000 units intramuscularly.

CASE 3.—Patient, V. W., a 58-year-old woman, was admitted July 12, 1945, to a hospital after an illness of one month's duration. The symptoms were sore throat, fever, and persistent headache. The patient had received 300,000 units of penicillin without improvement. On July 15, 1945, the patient became lethargic and developed signs of meningeal irritation, namely, rigid neck and positive Kernig and Brudzinski signs. There was also a slight left hemiparesis, and the left knee jerk was greater than the right. The lungs were clear, a short apical systolic heart murmur was heard, and the spleen was enlarged. Lumbar puncture yielded 20 c.c. of cloudy fluid from which pneumococcus type III was cultured. The patient was treated with large doses of penicillin intrathecally and intramuscularly and sulfadiazine

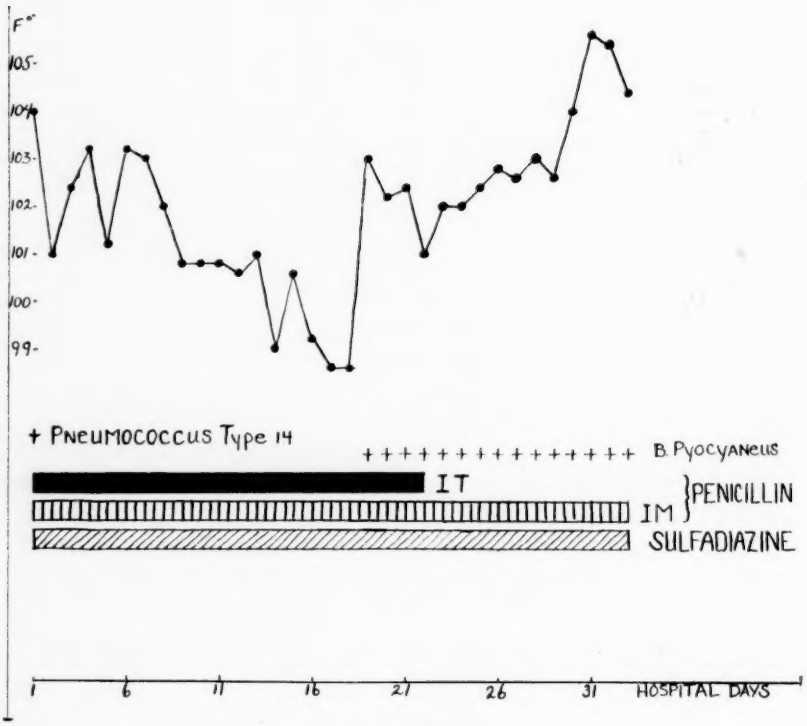

Fig. 2.—Case 2.

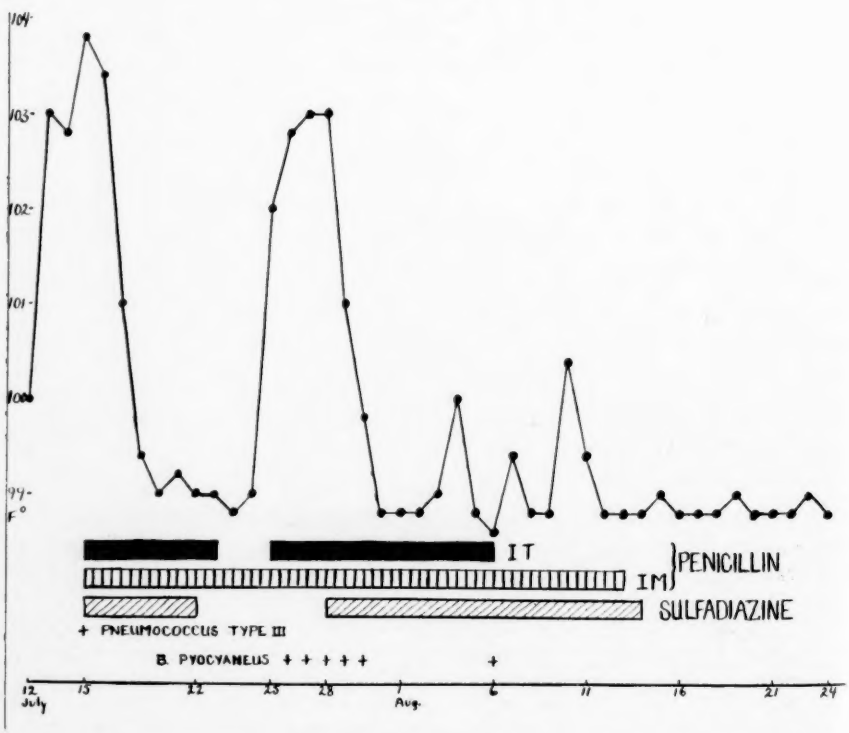

Fig. 3.—Case 3.

parenterally and orally. This treatment was continued for six days, after which the intrathecal penicillin and oral sulfadiazine were discontinued because of general improvement and sterile spinal fluid cultures.

Four days later the temperature rose to 102° F. (Fig. 3), and a cloudy spinal fluid was obtained from which *B. pyocyaneus* was cultured. This was confirmed by five subsequent positive cultures of the spinal fluid. For the succeeding 13 days oral sulfadiazine and intrathecal penicillin were given. Intrathecal penicillin therapy was then discontinued, but daily spinal drainage, intramuscular penicillin and sulfadiazine were continued until the temperature became normal. The patient left the hospital 48 days after admission, completely recovered.

CASE 4.—A. C., a 13-month-old infant, was hospitalized June 25, 1945, with a two-day history of fever and dehydration and convulsive tremors of the extremities for one day. Clinical signs of meningitis were present on admission, and type XVIII pneumococcus was cultured from the spinal fluid. No primary focus of infection could be found. Treatment consisted of large doses of sulfadiazine orally and penicillin intramuscularly for two weeks. For the first sixteen days of treatment the temperature fluctuated between 102 to 105° Fahrenheit. Intrathecal penicillin was given daily from July 10 to July 16 and from July 23 to August 3 because of persistent growth of pneumococcus from the spinal fluid. A middle ear infection became evident, and bilateral mastoidectomy was performed on July 31. From the right mastoid region *Staphylococcus aureus* was cultured, but no pneumococcus was found.

On Aug. 6, 1945, three days after the last intrathecal dose of penicillin, the patient's temperature rose to 102.6° F., and signs of meningeal irritation recurred. Cloudy spinal fluid was obtained from which *B. pyocyaneus* was cultured. No more intrathecal penicillin was used; however, the intramuscular dosage was continued for four more days, and oral sulfadiazine was continued in doses from 2.5 to 4.0 Gm. daily for the next nine days. The temperature gradually became normal and the patient completely recovered. *B. pyocyaneus* was recovered only on one occasion from the spinal fluid of this patient.

BACTERIOLOGIC INVESTIGATION

The day that the *B. pyocyaneus* was identified from the cultures of the spinal fluids of the two patients at Willard Parker Hospital, 20 vials of the penicillin that had been used for treatment on that ward were sent to the laboratory for study. These vials contained approximately 0.1 to 0.5 c.c. of clear penicillin solution diluted to 5,000 units per cubic centimeter. They were incubated at 37° C. without further dilution and showed no growth and were clear after forty-eight hours. However, after diluting the penicillin with 2 c.c. of beef heart infusion broth, by means of a syringe and needle inserted through the rubber stopper, *B. pyocyaneus* was recovered after twenty-four hours incubation at 37° C. or room temperature.

In order to check laboratory technique 16 additional vials, after use on that ward, were tested as follows: The aluminum band and rubber cap were removed, and 2 c.c. of broth were added from individual broth tubes by separate pipettes to each vial. All these vials of penicillin showed growth of *B. pyocyaneus* in twenty-four hours. The control broth tubes showed no growth after ninety-six hours of incubation at 37° Centigrade.

The method of preparing and administering the penicillin on the wards at our hospital was as follows: The penicillin was dissolved in 20 c.c. of saline drawn from a 500 c.c. "Sterisol flask." Individually wrapped syringes and needles, which were autoclaved, were used for the original dilution and for each

intrathecal dose. The rubber stopper was cleansed with alcohol before the needle was inserted. However, the intramuscular doses given at three-hour intervals were obtained by 2 c.c. syringes which were kept in a covered jar after having been boiled. Penicillin for both intramuscular and intrathecal doses for all patients receiving the drug were withdrawn from one vial until it was exhausted.

In order to determine the source of the *B. pyocyaneus* on the ward in which the infection occurred, penicillin from three fresh vials was cultured on the ward immediately after the diluent (saline) was added and also at the time of each (three- to four-hour intervals) intramuscular dose. Two-tenths cubic centimeter of penicillin from the syringe containing the intramuscular dose was added to 3 c.c. of broth before injection. The broth tubes were then incubated for ninety-six hours, and readings were made daily. Gram stains and subcultures into broth and on agar were made to identify the microorganisms found.

After ninety-six hours incubation no growth was observed in the cultures of the diluent (saline) and the penicillin immediately after dilution. However, *B. pyocyaneus* was found in all cultures of the penicillin which was drawn into 2 c.c. syringes for intramuscular injection.

At the same time the procedure outlined was also followed in the laboratory. One vial of penicillin was freshly diluted, and ten 0.2 c.c. samples at three-hour intervals were obtained by puncturing the disinfected rubber cap with a needle and a syringe. None of the cultures of the samples taken in the laboratory showed growth at the end of ninety-six hours of incubation.

In the laboratory the method of preparing and withdrawing the penicillin was the same as that on the ward, except that the 2 c.c. syringes used in the laboratory had been sterilized by dry heat in test tubes.

All the objects in the ward treatment room which could possibly be a source of contamination were cultured at the same time. The following results were obtained:

1. The jar in which the syringes were kept after boiling showed growth of *B. pyocyaneus.**

2. Syringes (barrel and plunger) in the jar, ready for use, showed growth of *B. pyocyaneus.*

3. Syringes (barrel and plunger), immediately after boiling, showed no growth.

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*The jars containing the syringes, the syringes, and the forceps used for taking them out were boiled in a fish kettle by the night nurses every twenty-four hours. The forceps and syringes were boiled also at other times if they came in contact with contaminated material. The jars were kept on the "dressing" table and were closed at all times, except when the syringes were being removed. The syringes were boiled unassembled, and the barrel and plunger were kept together by a rubber band during sterilization and kept in the jar. The sterilized forceps were kept in 0.4 per cent solution of lysol while not in use. There was, however, no way of checking whether during the wartime shortage of nurses and pressure of work this technique always had been carried out as outlined and whether the jars had actually been sterilized every night. It is probable that some of the jars had become contaminated through some breach of technique and that freshly sterilized syringes became in turn contaminated when placed in the jars.

The only other patient treated at the same time was a 6-year-old boy who was given penicillin intramuscularly. He had a rise in temperature at the same time as the other two patients (Cases 1 and 2). No local abscess appeared at any time, and his recovery was uneventful.

4. The sterilizer used for boiling the syringes showed no growth.

5. The box in which the penicillin was kept in the refrigerator as well as the caps on 2 vials in the box showed a few colonies of *Staphylococcus albus.*

6. Top and shelves and tray on the treatment table showed *Staph. albus* and *Aerobacter aerogenes.*

7. The window sill showed few *Staph. albus* and *A. aerogenes.*

8. Lysol and stock 70 per cent alcohol and the alcohol cotton wipes showed no growth.

From these studies on the ward in which the infection occurred, we were able to conclude that the syringes after being sterilized by boiling were contaminated by being kept in a jar harboring *B. pyocyaneus.* These syringes, when used to withdraw the intramuscular doses of penicillin, contaminated the drug.

After the source of contamination was traced in the ward in question, vials having some residual penicillin, the jars in which the syringes ready for use were kept, and the barrels and plungers from all the other wards in the hospital were cultured. Of the 21 vials tested, 3 vials showed growth of *B. pyocyaneus* and 4 of *Staph. albus* and of *A. aerogenes.* Two syringes showed growth of the aerobacter and two others *Staph. albus.*

A total of 61 vials of penicillin were cultured from all wards; of these, 41 showed growth of *B. pyocyaneus,* 39 from the ward where the accidental infections occurred. The vials of penicillin containing 5,000 units per cubic centimeter from which either the *B. pyocyaneus* or the *A. aerogenes* was obtained were clear. The vials that showed contamination were not from any particular lot number or from only one commercial biologic laboratory.

To insure absolute sterile conditions for the dilution and administration of penicillin, the following procedure was adopted at the Willard Parker Hospital:

1. Individual 20 c.c. ampules of saline are used to dilute the penicillin.

2. All needles and 2 c.c. syringes are assembled and placed in test tubes, and large syringes are individually wrapped and then all equipment sterilized in the autoclave.

3. Caps of the penicillin vials are washed with individual iodine and alcohol swabs before puncturing.

4. Individual vials of penicillin are used for each patient and are labeled with the name, date, and ward number.

DISCUSSION

Penicillin solutions used on the wards in three hospitals became contaminated with *B. pyocyaneus.* As the solutions remained clear they were considered sterile and were used therapeutically. The intramuscular injections produced no abscesses at the site of inoculation; the intrathecal injections, however, resulted in *B. pyocyaneus* meningitis in four patients with death in two cases.

Although penicillin in high concentrations may inhibit the active growth phase of either *B. pyocyaneus* or *A. aerogenes,* it has been established by Fleming[7] and Abraham and associates[8] that it is not bacteriocidal for these microorganisms. It is therefore important to maintain absolutely sterile technique in the preparation and administration of penicillin. This is especially important, since the penicillin shows no visual evidence of contamination and remains clear even at a concentration of 5,000 units per cubic centimeter and only becomes cloudy after it is diluted to contain less than 1,000 units per cubic centimeter.

SUMMARY

1. Secondary *B. pyocyaneus* meningitis following intrathecal penicillin treatment for pneumococcus meningitis was observed in four patients, two of whom died.

2. The source of the *B. pyocyaneus* was traced in two of the cases to penicillin solutions contaminated by syringes used to withdraw the drug for injection.

3. As penicillin solutions containing 5,000 units per cubic centimeter show no evidence of contamination, absolutely sterile technique in the preparation and administration of penicillin must be observed.

We wish to extend our thanks to Dr. Vera B. Dolgopol and Dr. Jerome L. Kohn, for their aid in preparation of this paper, and to Miss Ruth Gosling, for her assistance in the bacteriologic diagnosis.

REFERENCES

1. Schlagenhaufer, F.: Pyocyaneus Infection After Spinal Anesthesia, Centralbl. f. Bakt. (Abteilung 1) **59:** 385-387, 1911.
2. Sonnenschein, C.: Fatal Meningitis After Lumbar Puncture, Deutsche med. Wchnschr. **49:** 881-882, 1923.
3. Levy, I. I., and Cohen, A. E.: Pyocyaneus Meningitis After Lumbar Puncture, J. A. M. A. **85:** 1968-1969, 1925.
4. Evans, F. L.: Primary Meningitis Caused by Pseudomonas Aeruginosa (Bacillus Pyocyaneus), M. Rec. **144:** 111, 172, 1936.
5. Wise, R. A., and Musser, J. H.: Bacillus Pyocyaneus Meningitis; Report of 6 Cases, New Orleans M. & S. J. **92:** 145-151, 1939.
6. Kerman, W. Z., Perlstein, M. A., Levinson, A.: Bacillus Pyocyaneus Meningitis Following Pneumoencephalography, Am. J. Dis. Child. **65:** 912-915, 1943.
7. Fleming, A.: On the Specific Antibacterial Properties of Penicillin and Potassium Tellurite; An Incorporating Method of Demonstrating Some Bacterial Antagonisms, J. Path. & Bact. **35:** 831-842, 1932.
8. Abraham, E. P., Chain, E., Fletcher, C. M., Gardner, A. D., Heatley, N. G., Jennings, M. A., and Florey, H. W.: Further Observations on Penicillin, Lancet **2:** 177-188, 1941.

THE TWO-DOSE DEXTROSE TOLERANCE TEST IN THE DIAGNOSIS OF DIABETES MELLITUS

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THE standard single dose dextrose tolerance test, first used by Jacobson,[1] has not proved entirely satisfactory for the diagnosis of diabetes mellitus. The procedure is not only time-consuming and discomforting but also lacking in sensitivity and specificity.[2-4] Moreover, the results of the test are influenced significantly by the composition of the antecedent diet.[5-9]

To vitiate the deficiencies in the standard test, several modifications have been proposed. One of these, in which two doses of dextrose are given one to two hours apart, was suggested by Staub[10] and Traugott.[11] In the hands of Malmros[12] and Julesz[13] this procedure seemed none too satisfactory, and so fell into disuse.

The two-dose test was revived and further modified by Exton and Rose,[14] who shortened the interval between the first and second dose of dextrose to one-half hour and who abbreviated the duration of the procedure to one hour.

The two-dose one-hour test subsequently has gained considerable popularity. It has been used in the Out-Patient Diabetes Clinic of Strong Memorial Hospital* since 1938. We propose herein to summarize our experience with the test, which in clinic practice has been timesaving, economical, and satisfactory for the diagnosis of diabetes mellitus. Of special interest are the data concerning the value of an additional sugar determination made two hours from the beginning of the test.

Technique of the Test.—All individuals tested were ambulant patients in the Out-Patient Department of Strong Memorial Hospital. Each was instructed to take neither food nor insulin from the time of the evening meal until the test had been completed the following morning. No attempt was made to control the diet preceding the test.

The sugar solution was prepared by dissolving 100 Gm. of dextrose in 650 c.c. of water. The resulting 15 per cent solution was divided into two equal portions.

After the patient had voided, a specimen of venous blood was withdrawn and the first portion of the dextrose solution given. Two minutes were allowed for its ingestion. Thirty minutes later, blood and urine specimens were obtained and the second portion of the dextrose solution given. Third and fourth specimens of blood and urine were obtained one hour and two hours, respectively, after the first dose of dextrose solution had been given.

The Physiologic Basis of the Two-Dose Test.—The two-dose dextrose tolerance test is based upon sound physiologic principles. The first of these, known as Allen's[15] law of paradoxic dextrose utilization, is the fact that the more dextrose ingested by a normal individual the more is utilized. The patient with diabetes mellitus, on the other hand, limited in his ability to cope with carbohydrates, develops hyperglycemia and usually glycosuria after the ingestion

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of sizable amounts of dextrose. The second principle, the Hamman-Hirschman[16] phenomenon, is that when dextrose is given to nondiabetic persons in two increments the hyperglycemia which follows the second dose is less than that produced by the first. The diabetic patient, less adept in removing excess carbohydrate from the blood, responds with increased hyperglycemia to repeated doses of dextrose.

Advantages and Disadvantages of the Two-Dose Test.—The principal advantages of the two-dose test are its brevity, simplicity, and economy. The shorter duration of the test diminishes the effect of factors such as hunger, fatigue, and impatience. Fewer venipunctures and fewer chemical determinations are necessary than with the standard test.

There is some reason to believe that the composition of the antecedent diet distorts the results of the two-dose test less than those of the standard one-dose procedure. Presumably, this is because the first increment of dextrose acts as a buffer against whatever depletion of liver glycogen may have occurred before the test is performed.

The results obtained by groups of investigators studying the influence of the antecedent diet upon the two-dose one-hour test are at variance. Exton and Rose state that moderate variations in the diet do not influence the test. Sweeney, Muirhead, and Allday,[17] drastically altering the composition of their subjects' diets, found effects they considered significant, and Langner and Fies[18] state that "moderate as well as extreme manipulation of the diet is capable of altering the Exton-Rose glucose tolerance from normal to the diabetic type." Wayburn and Gray,[19] however, found the two-dose test "relatively free from the influence of fairly marked changes in the preceding diet and therefore superior to the one-dose test when the preceding diet is not known."

A disadvantage of the test noted by Young[20] is that when dextrose absorption is unusually rapid, the two-dose test gives an erroneous picture of the true status of the patient's carbohydrate tolerance. In our experience these individuals may be identified by prolongation of the two-dose test to include a two-hour specimen.

Another disadvantage of the test, although one seldom encountered, is that nervousness may delay passage of the ingested dextrose from the stomach into the intestine. Gastric motility varies considerably from subject to subject,[21, 22] and, in a few, delay in the emptying time of the stomach may retard the hyperglycemic response, a factor which merits consideration because of the brief time interval involved.

Comparison of the Two-Dose Test With the Standard One-Dose Procedure.—No extensive comparison between the standard one-dose test and the two-dose procedure is available. Kelly, Beardwood, and Fowler[23] found the two tests in close agreement and cite two cases out of fifty in which the two-dose test was found to be superior. Brown[24] also has compared the two procedures and found them equally accurate. Langner and Dewees,[25] who studied 160 patients, believe that the conventional one-dose test is the more specific of the two, although they consider the Exton-Rose test somewhat more sensitive.

Interpretation of the Two-Dose Test.—The most cogent reason why the two-dose procedure has not displaced the standard dextrose tolerance is that there has not been complete agreement as to the interpretation of the results.

The criteria of Exton and Rose[26] and those of Gould, Altshuler, and Mellen[27] are at variance. The most extensive study of the test is that of Matthews, Magath, and Berkson,[28] and most subsequent workers have agreed with their interpretation.

TABLE I. PROPOSED CRITERIA FOR INTERPRETATION OF EXTON-ROSE TEST

1. Exton and Rose[26]
   - A. Normal tolerance curve:
     - (1) Normal fasting blood sugar
     - (2) Rise in blood sugar during first half hour not to exceed 75 mg. per 100 c.c.
     - (3) Rise in blood sugar during second half hour not to exceed 5 mg. per 100 c.c.
   - B. Diabetic tolerance curve:
     - (1) A more or less steep curve of not less than 10 mg. of blood sugar following the second dose of dextrose.
     - (2) Relation of blood and urine sugar values to the severity of the disease.
2. Gould, Altshuler, and Mellen[27]
   - A. Normal tolerance curve:
     - (1) Fasting blood sugar less than 120 mg. per 100 c.c.
     - (2) The one-half hour level is less than 50 mg. per 100 c.c. above the fasting value.
     - (3) The level of the blood sugar at one hour is less than 30 mg. above the one-half hour level.
   - B. Diabetic tolerance curve:
     - (1) Fasting blood sugar of 120 mg. or more.
     - (2) One-half hour level of 50 mg. or more above the fasting.
     - (3) One hour level of 30 mg. or more above the one-half hour level.
     - (4) Two of these three criteria necessary for diagnosis.
3. Matthews, Magath, and Berkson[28]
   - A. All individuals with blood sugar level at one hour of less than 158 mg. per 100 c.c. considered nondiabetic, and all over 180 mg. per 100 c.c. are considered diabetic. Those between 158 and 180 mg. are nondiagnostic.

With the criterion of Matthews, Magath, and Berkson, we also agree. In our experience the one-hour blood sugar value is the most reliable indication of the presence or absence of diabetes mellitus. We believe, however, that implicit reliance on a single chemical determination may be misleading. No test of physiologic function as complex as is the regulation of the blood sugar can be expected to be definitive. That this is true of the two-dose dextrose tolerance test is apparent from the material upon which this study is based.

Each case should be viewed in its entirety, and special consideration should be given to the following: the existence of diabetes in members of the patient's family, the presence of obesity or of diseases of organs concerned with carbohydrate metabolism (liver, hypophysis, thyroid), and the effect of infections upon the patient's carbohydrate tolerance.

ANALYSIS OF CASES

In the present study a statistical analysis has been made of the results of the two-dose test performed on twenty-six normal persons, eighty-two in whom a diagnosis of diabetes mellitus was considered to have been established, seven with renal glycosuria, and six subjects whose dextrose tolerance curves are considered borderline or doubtful.

The diagnosis of diabetes mellitus was established by considering the following: the symptoms of which the patient complained, a history of the

TABLE II. AVERAGE BLOOD SUGAR READINGS

	NUMBER OF CASES	FASTING		
		MEAN	RANGE	δ OF MEAN
Nondiabetic				
Normal	26	77.4	61 to 94	8.5
Renal glycosuria	7	83.1	72 to 91	6.9
Diabetic				
No insulin	49	138.2	75 to 334	48.8
Insulin at onset only	11	153.5	96 to 233	43.6
Insulin treatment	22	179.7	76 to 315	66.8
Total diabetic	82	151.4	75 to 334	55.8

disease in other members of the family, the absence of other conditions associated with hyperglycemia, the degree of glycosuria, the patient's reaction to infections, and the results of the two-dose test.

Of the eighty-two patients with diabetes mellitus: twenty-two required insulin daily; forty-nine were regulated satisfactorily without insulin; and

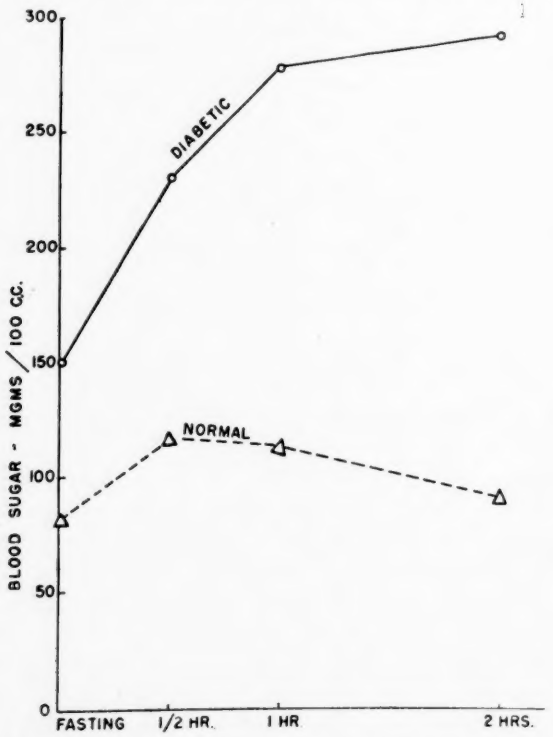

Fig. 1.—Average blood sugar readings with the two-dose test.

eleven were given insulin at the onset but subsequently were able to dispense with the injections as their condition improved from weight reduction, dietary regulation, and control of infections.

No attempt was made to subclassify the group of insulin-treated diabetic patients according to the amount of insulin required. Nor did there seem any

ITH TWO-DOSE DEXTROSE TOLERANCE TEST

ONE-HALF HOUR			ONE HOUR			TWO HOURS		
MEAN	RANGE	δ OF MEAN	MEAN	RANGE	δ OF MEAN	MEAN	RANGE	δ OF MEAN
115.6	84 to 135	14.4	113.9	74 to 150	21.5	91.9	56 to 125	19.9
115.8	106 to 124	9.8	112.2	91 to 127	14.6	101.0	79 to 125	13.8
222.4	99 to 475	67.7	266.8	180 to 500	63.3	268.8	135 to 500	87.1
212.9	96 to 333	72.9	283.2	222 to 377	77.4	291.4	182 to 421	109.9
260.6	118 to 385	70.6	307.1	170 to 444	77.5	348.4	136 to 500	102.0
231.4	96 to 475	70.5	279.8	170 to 500	70.5	295.6	135 to 500	99.7

special virtue in trying to evaluate the "severity" of diabetes from the results of the dextrose tolerance test alone.

The results of the tests, expressed as average readings for each group, are shown in Table II and represented graphically in Fig. 1.

TABLE III. ANALYSIS TO DETERMINE STATISTICAL VALIDITY OF CRITERIA FOR INTERPRETATION OF TWO-DOSE TEST

	NORMAL		DIABETIC		δ OF DIFFERENCE OF MEANS	$X = M_1 - M_2$	$\frac{X}{\delta}$
	MEAN	δ OF MEAN	MEAN	δ OF MEAN			
Fasting							
Height of fasting blood sugar	77.4	8.5	151.4	55.8	55.6	74.0	1.33
One-half hour							
Height of one-half hour blood sugar	115.6	14.4	231.4	70.5	71.8	115.8	1.61
Milligrams change; fasting, one-half hour	38.2	13.4	80.0	43.1	45.0	41.8	0.93
Percentage change; fasting, one-half hour	49.3	19.2	52.8	41.7	45.9	3.5	0.08
One hour							
Height of one hour blood sugar	113.9	21.5	279.8	70.5	73.7	165.9	2.25
Milligrams change; one-half to one hour	−1.7	19.5	48.4	65.1	67.9	50.1	1.36
Percentage change; one-half to one hour	−1.5	17.0	20.9	21.4	27.3	22.4	0.82
Milligrams change; fasting, one hour	36.5	19.5	128.4	51.8	55.4	91.9	1.66
Percentage change; fasting, one hour	47.2	25.6	84.8	69.2	73.7	37.6	0.51
Two hours							
Height of two hours blood sugar	91.9	19.9	295.6	99.6	102.0	203.7	2.00
Milligrams change; one hour to two hours	−22.0	16.6	15.8	49.0	51.7	37.8	0.73
Percentage change; one hour to two hours	−19.3	14.3	5.6	17.4	22.6	24.9	1.10
Milligrams change; one-half hour to two hours	−23.7	20.6	64.2	58.5	62.1	87.9	1.42
Percentage change; one-half hour to two hours	−20.5	18.7	27.7	28.2	33.9	48.2	1.42
Milligrams change; fasting, two hours	14.5	16.5	144.2	123.2	124.4	129.7	1.04
Percentage change; fasting, two hours	18.7	22.2	95.2	51.8	75.2	76.5	0.98

Consideration of the mean values alone indicates that in the average case, the two-dose procedure readily separates the diabetic patient from the nondiabetic person. Standard deviations of the mean values, however, tend to be large, especially in the diabetic groups. Further statistical analysis therefore was made in an attempt to determine which criteria are most reliable. The results of this analysis are summarized in Table III.

The data of Table III indicate that the height of the one-hour blood sugar reading is the most reliable criterion. The height of the two-hour blood sugar appears to be the next most valid differential point. The x/δ values of the other calculations are below 2.0, and therefore cannot be considered statistically significant.

From this analysis, we believe the data in Table IV to represent the most important considerations in attempting to differentiate the normal from the diabetic by the two-dose dextrose tolerance test.

TABLE IV. CRITERIA FOR DIFFERENTIATION OF NORMAL PERSONS FROM PATIENTS WITH DIABETES MELLITUS

	BLOOD SUGAR READINGS		
	NORMAL	DOUBTFUL	DIABETIC
One hour	Below 150	150 to 170	Above 170
Two hours	Below 125	125 to 135	Above 135

TABLE V. CORRECTNESS OF PROPOSED DIAGNOSTIC CRITERIA

	TOTAL NUMBER OF CASES*	EXTON AND ROSE		GOULD AND ASSOCIATES		MATTHEWS AND ASSOCIATES		CONNER AND REYNOLDS	
		NUMBER	PERCENTAGE	NUMBER	PERCENTAGE	NUMBER	PERCENTAGE	NUMBER	PERCENTAGE
Normal	29	20	69	21	72	27	93	29	100
Diabetic	85	78	92	77	91	82	96	84	99

*Includes six borderline cases summarized in Table VI.

All of the cases, both normal and diabetic, summarized in Table II meet both of the criteria set forth previously. The number of correct diagnoses which would have been made had the criteria of others alone been relied upon is shown in Table V.

ANALYSIS OF BORDERLINE CASES

There remain nine cases in which the diagnosis of diabetes mellitus could neither be readily established nor definitely excluded. These cases are detailed in Table VI.

In Cases 1 to 3, there was clinical evidence of hepatic or pituitary disease.

Cases 4 and 5 are considered diabetes mellitus, although the one-hour reading leaves some doubt as to this diagnosis. The low blood sugar at one hour may represent delay in gastric emptying time. Cases 6 to 8 probably represent instances of unusually rapid dextrose absorption by nondiabetic persons, as suggested by Young. Technical error in the blood sugar determinations cannot be ruled out. Case 9 is unique in our experience. This patient, when studied in the Out-Patient Department, showed no evidence of diabetes, although two years previously he was said to have had glycosuria during

TABLE VI. SUMMARY OF BORDERLINE CASES

CASE	PATIENT	CLINICAL DIAGNOSIS	TWO-DOSE TEST	DIAGNOSIS BY CRITERIA OF: EXTON AND ROSE	GOULD AND ASSOCIATES	MATTHEWS AND ASSOCIATES
1	V. L.	Cirrhosis of liver	95-143-182-118	Diabetes	Indeterminate	Diabetes
2	C. J.	Cirrhosis of liver	87-133-163 65-115-160-156	Diabetes	Normal	Indeterminate
3	R. M.	Pituitary basophilism	80-161-179-145	Diabetes	Normal	Indeterminate
4	B. S.	Diabetes	84-118-164-160	Diabetes	Indeterminate	Indeterminate
5	A. V.	Diabetes	74-111-140-160 86-95-168-165	Diabetes	Normal Indeterminate	Normal Indeterminate
6	N. P.	Rapid absorption	100-208-135-90	Normal	Indeterminate	Normal
7	E. J.	Rapid absorption	93-160-184-83	Diabetes	Indeterminate	Diabetes
8	L. B.	Rapid absorption	87-139-175-118	Diabetes	Diabetes	Indeterminate
9	P. M.	Diabetes	82-111-127	Diabetes	Normal	Normal

hospitalization for drainage of a palmar abscess. Six months later he was admitted to the hospital with pyelonephritis and in the presence of infection again became frankly diabetic with hyperglycemia and glycosuria, requiring the use of insulin.

DISCUSSION

As with other laboratory procedures, the two-dose dextrose tolerance test should be used only to supplement other clinical considerations in attempting to differentiate the diabetic from the nondiabetic patient.

Properly interpreted, the test is a valuable procedure for making this differentiation. Greatest reliance should be placed upon the height of the blood sugar at one hour. However, it is distinctly helpful to see the complete curve and to correlate the readings with other factors. The one-half hour reading contributes little to the differentiation, but the two-hour reading has proved of great value in borderline cases. The results of the test should be interpreted with reserve in the presence of diseases of the liver, thyroid, or pituitary gland.

CONCLUSIONS

1. The two-dose dextrose tolerance test is based on sound physiologic principles and is sufficiently sensitive and specific to make it a valuable procedure for the diagnosis of diabetes mellitus in clinic and office practice.

2. The height of the one-hour blood sugar is the most reliable criterion for differentiating the normal person from the diabetic patient.

3. Determination of the blood sugar at one-half hour contributes little to the differentiation and may well be deleted from the procedure. The two-hour reading, however, is reliable and should be included in all doubtful cases.

4. In a few cases no definitive diagnosis can be established from the results of the dextrose tolerance test alone. These cases should be viewed in their entirety, and special consideration should be accorded the clinical picture.

5. When the one-hour blood sugar reading is within the diabetic range and the two-hour reading normal, excessively rapid dextrose absorption should

be suspected. When the one-hour blood sugar reading is normal and the two-hour specimen indicative of diabetes, delayed gastric emptying is to be considered.

REFERENCES

1. Jacobsen, A. T. B.: Untersuchungen über den Einfluss verschiedener Nahrungsmittel auf den Blutzucker bei normalen, zuckerkranken und graviden Personen, Biochem. Ztschr. **56**: 471, 1913.
2. Gray, H.: Blood Sugar Standards; Normal and Diabetic Persons, Arch. Int. Med. 31: 241, 1923.
3. Mosenthal, H. O.: Interpretation of Sugar Tolerance Tests; Common Occurrence of Renal Glycosuria, M. Clin. North America **9**: 549, 1925.
4. Myers, G. B., and McKean, R. M.: The Oral Glucose Tolerance Test: A Review of the Literature, Am. J. Clin. Path. **5**: 299, 1935.
5. Southwood, A. R.: Some Effects of Carbo-Hydrate Deprivation Upon Pancreatic Function, M. J. Australia **2**: 460, 1923.
6. Greenwald, I., Gross, J., and Samet, J.: The Nature of Sugar in Normal Urine; The Sugar Excretion Upon Various Diets and the Influence of Diet Upon Glucose Tolerance With Some Remarks on the Nature of the Action of Insulin, J. Biol. Chem. **62**: 401, 1924.
7. Sweeney, J. S.: Dietary Factors That Influence the Dextrose Tolerance Test, Arch. Int. Med. **40**: 818, 1927.
8. McCullough, E. P., and Johnston, C. R. K.: Manipulation of Glucose Tolerance by Diet, Am. J. M. Sc. **195**: 773, 1938.
9. Conn, J. W.: Interpretation of the Glucose Tolerance Test. The Necessity of a Standard Preparatory Diet, Am. J. M. Sc. **199**: 555, 1940.
10. Staub, H.: Bahnung im intermediaren Zuckerstoffwechsel, Biochem. Ztschr. **118**: 93, 1921.
11. Traugott, K.: Ueber das Verhalten des Blutzuckerspiegels bei wiederholter und verschiedener Art enteraler Zuckerzufuhr und dessen Bedeutung für die Leberfunktion, Klin. Wchnschr. **1**: 892, 1922.
12. Malmros, H.: Study of Glycosuria, With Special Reference to Interpretation of Incidental Finding of Positive Reduction Test, Acta med. Scandinav. (supp. no. 27), pp. 1-309, 1928.
13. Julesz, M.: Die Bewartung der doppelten Glucosebelastungsversuche, Ztschr. f. d. ges. exper. Med. **79**: 153, 1921.
14. Exton, W. G., and Rose, A. R.: Diabetes as a Life Insurance Selection Problem, Proc. Life Insur. M. Dir. America **18**: 252, 1932.
15. Allen, F. M.: Studies Concerning Glycosuria and Diabetes, Boston, 1913, W. M. Leonard, p. 22.
16. Hamman, L., and Hirschman, I. I.: Studies on Blood Sugar. IV. Effects on the Blood Sugar of the Repeated Ingestion of Glucose, Bull. Johns Hopkins Hosp. **30**: 306, 1919.
17. Sweeney, J. S., Muirhead, J. J., and Allday, L. E.: Observations on the One Hour Two Dose Dextrose Tolerance Test, Am. J. Clin. Path. **7**: 482, 1937.
18. Langner, P. H., and Fies, H. L.: The Influence of Antecedent Diet Upon the Exton-Rose One-Hour, Two-Dose Glucose Tolerance Test, Am. J. Clin. Path. **11**: 41, 1941.
19. Wayburn, E., and Gray, H.: The Two-Dose Glucose Tolerance Test, Am. J. M. Sc. **204**: 823, 1942.
20. Young, C. J.: Sugar Tolerance Test of Exton and Rose, Lancet **2**: 1367, 1937.
21. Kasabach, H. H.: Effect of Ingestion of Water and of Dextrose Solution on Emptying Time of Normal Stomach, Arch. Int. Med. **48**: 1237, 1931.
22. Beeler, C., Bryan, A. W., Catheart, E. P., and Fitz, R.: An Improved Alimentary Glucose Tolerance Test, J. Metabolic Research **1**: 549, 1922.
23. Kelly, H. T., Beardwood, J. T., Jr., and Fowler, K.: The Value of the One-Hour Two-Dose Glucose Tolerance Test (Exton and Rose) in the Early Diagnosis of Diabetes Mellitus, Am. J. Clin. Path. **5**: 411, 1935.
24. Brown, A. J.: Comparison of Relative Values of Certain Glucose Tolerance Tests, M. Bull. Vet. Admin. **16**: 152, 1939.
25. Langner, P. H., and Dewees, E. J.: Instances of Disagreement in the Results of Two Types of Oral Glucose Tolerance Tests, Am. J. M. Sc. **204**: 85, 1942.
26. Exton, W. G., and Rose, A. R.: The One-Hour Two-Dose Dextrose Tolerance Test, Am. J. Clin. Path. **4**: 381, 1934.
27. Gould, S. E., Altshuler, S. S., and Mellen, H. S.: The One-Hour Two-Dose Glucose Tolerance Test in the Diagnosis of Diabetes Mellitus, Am. J. M. Sc. **193**: 611, 1937.
28. Matthews, M. W., Magath, T. B., and Berkson, J.: The One-Hour Two-Dose Dextrose Tolerance Test (Exton-Rose Procedure): Diagnostic Significance, J. A. M. A. **113**: 1531, 1939.

A DETAILED REPORT ON THE WEIGHTS AND WEIGHT LOSSES OF TWENTY-FOUR MEN IN SANTO TOMAS INTERNMENT CAMP

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ALTHOUGH there have been a number of reports on the nutrition in various prison and internment camps of World War II, there has been to my knowledge no detailed report on a group by one who knew the individuals and their condition intimately. It was therefore considered to be of value to report the following data.

In Table I are presented the weights of twenty-four men civilian internees of Santo Tomas Internment Camp in Manila, P. I., thirty-six months after their internment and two weeks before liberation on Feb. 3, 1945. The classification of body types was made by the author who had lived in either the same or adjoining rooms with these men for eighteen months or more. The ideal weights were taken from tables compiled by the Statistical Bureau of the Metropolitan Life Insurance Company (Wohl[1]). This particular group of men cannot be said to be entirely typical of the camp population, as they were the more healthy individuals remaining from two rooms after the other members not so fortunate had been transferred to quarters considered to be more suitable for those in a poor state of health. In fact this group, in general, had been able to obtain considerable amounts of supplementary food in addition to the camp ration. For example the author had been able to add about 3 ounces of canned meat daily to his diet and about 1 ounce of salad oil during the last five months. This, in addition to consuming large amounts of peanut butter as long as it was available (until about May, 1944) and gorging himself with rice as long as it was available (until about Feb. 1, 1944), allowed him to keep his weight at 93 per cent of the ideal. However, in spite of this, his blood pressure was 90 mm. Hg instead of a normal 127 mm. Hg; pulse, 48 to 50 instead of a normal 70; red blood cells, 3.25 millions per cubic millimeter instead of 5.5 millions per cubic millimeter; and the hemoglobin about 55 per cent. At this time a blood pressure of 100 mm. Hg and a red blood cell count of 4 millions per cubic millimeter or over was considered exceptional in the camp population.

Perhaps it should be added that the low red blood cell and hemoglobin values of the author were no doubt in part due to three donations for transfusion of about 500 c.c. each given in the latter part of 1943 which dropped the red blood cells to 3.5 millions per cubic millimeter. Early in 1944 a treatment with injectible liver preparations plus 70 Gm. of peanut protein daily raised this to 4.25 millions per cubic millimeter in six weeks. Several months

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TABLE I. DETAILED DATA ON WEIGHTS AND WEIGHT LOSSES OF TWENTY-FOUR MEN INTERNEES OF SANTO TOMAS INTERNMENT CAMP, MANILA, P. I., AFTER THIRTY-SIX MONTHS OF INTERNMENT AND TWO WEEKS BEFORE LIBERATION

NO.	SUBJECT	AGE (YEARS)	FRAME TYPE†	HEIGHT (INCHES)	WEIGHT						
					PREWAR (POUNDS)	IDEAL (POUNDS)	FINAL (POUNDS)	LOSSES (POUNDS)	FINAL AS PER CENT OF		PREWAR AS PER CENT OF IDEAL
									PREWAR	IDEAL	
1	P. K.*	18	L	67	--	135	93	--	--	69	--
2	L. C.	33	M-H	69	160	160	130	30	81	81	100
3	B. H. B.	34	M-H	68	165	156	145	20	88	93	106
4	J. S.	34	H	67	185	155	125	60	68	81	119
5	P. E. M.	35	L	68	135	140	113	23	84	81	96
6	R. H.	35	M	71	170	163	123	47	72	75	104
7	R. B. D.	36	M-H	72	178	173	145	33	81	84	103
8	L. C.	36	L	72	145	158	135	10	93	86	92
9	A. E. L.	36	H	72½	190	185	150	40	79	81	103
10	D. C. B.	37	L	68	148	142	110	38	74	77	104
11	W. D.	38	M	71	162	162	114	48	70	70	100
12	G. M.	38	L	70½	140	155	110	30	79	71	90
13	R. G.‡	39	L	69	170	146	98	72	58	67	117
14	L. B.	39	L-L	73	153	157	122	31	80	78	98
15	D. C.	40	M-L	68	148	147	125	23	84	85	101
16	B. P.	40	M	68	190	150	127	63	67	85	127
17	S. C.	43	L	70	157	150	114	43	73	76	105
18	F. V. K.	47	M	68½	185	156	137	48	74	88	119
19	M.	55	M	67	160	146	114	46	71	78	110
20	R.‡	58	L	70	157	150	120	37	76	80	105
21	N. G.	60	L	70	186	150	122	64	65	81	124
22	H.	65	L	68	178	142	115	63	65	81	125
23	B. H. B.	65	H-H	66½	187	160	141	46	75	88	117
24	S. K.‡	74	M	70	178	158	127	51	71	80	113
	Averages				166	155	124	42	75	80	108

*Excluded from averages.

†L, M, and H refer to light, medium, and heavy frame types, respectively.

‡Slight edema in lower extremities.

later, when the count had again fallen off considerably, a second series of liver injections without the supplementary protein (no longer available) had no effect on the red blood cell count.

Keys and co-workers,[2] in experiments with thirty-four men, found that edema had developed in nearly all cases in a six months' starvation period during which the subjects lost 25 per cent of their weight and were on a protein level of 49 Gm. derived from vegetable sources. It therefore seems rather remarkable that so few of our group showed edema at this time, for as is shown in Table I we had lost on an average of 25 per cent in weight although over a longer period of time. As indicated in Table I only Subjects 13, 20, and 24 showed definite edema at this time. In the two weeks which ensued after these data were collected and before liberation, Subjects 1, 5, 10, 21, and 22 also showed traces of edema. Our calorie level from the camp ration had been less than 1,000 for over five months and the protein level at 20 to 30 Gm. obtained chiefly from rice and corn with a few beans, sweet potatoes, and negligible amounts of meat. Even the author, whose diet was supplemented by about 3 ounces of meat daily, received only 40 to 50 Gm. of protein daily. It seems possible that we had gradually become adapted to a low level of protein and general metabolism, as the blood pressure and pulse rates would suggest.

TABLE II.* AVERAGE WEIGHTS OF VARIOUS AGE GROUPS AT SANTO TOMAS INTERNMENT CAMP, MANILA, P. I., THIRTY-SIX MONTHS AFTER INTERNMENT AND TWO WEEKS BEFORE LIBERATION

SUBJECTS	AGE	NUMBER OF PERSONS	AVERAGE WEIGHTS			
			PREWAR (POUNDS)	36 MONTHS LATER (POUNDS)	LOSSES (POUNDS)	PER CENT OF PREWAR
Men	19 to 40	508	166	124	42	75
Men	41 to 60	555	175	122	53	70
Men	Over 60	433	177	119	58	67
Camp totals		1,506	172	121	51	70
Women	19 to 40	667	125	101	24	81
Women	41 to 60	448	135	100	35	74
Women	Over 60	119	149	96	53	64
Camp totals		1,232	132	100	32	76

*Official Records of Santo Tomas Internment Camp. Unpublished.

It is interesting to note that our camp average of 121 pounds (Table II) for men was from 10 to 15 pounds below that of 4,618 prisoners of war freed in the Far East and reported by the military authorities.[3]

Table II also bears out the general observation made by the author that the women fared considerably better than the men. Their weight at this time was 76 per cent of normal as against 70 per cent for the men. This was probably largely due to the fact that they received the same food rations as the men in spite of the fact that their requirements were considerably less. It is also noticeable (Table II) that the older and more obese individuals lost more weight and reached a final lower weight than the younger people. This was probably due to several factors. In the first place, the younger people worked more actively, especially around the kitchen, and thus received more food in

many cases. The younger ones were more vigorous and more able to produce extra food in gardens of their own or to do work for other individuals in camp and thus obtain either directly or indirectly more food. The younger people also were not so particular about their food and regardless of former food habits ate rice, unappetizing stew, or anything else that could be had, while many of the older people, in the earlier days when considerable amounts of food were thrown away daily, refused to eat heartily and thus started to decline long before it was necessary.

The unusual case of an extremely obese individual (B. C., Negro) who entered camp weighing 365 pounds and dropped to 148 pounds, a loss of 217 pounds or 59.4 per cent of his total weight, is also noted.[4]

It might be of interest to point out that, in general, this group of men had lived for extended periods in the tropics. Several had been born in the Islands and except for visits and education abroad had spent their lives there. Two others, Subjects 24 and 25, had spent approximately forty years each in the Islands. Many others had lived there from five to ten years. The relative condition of these individuals suggests that the old idea of the unhealthy climate of the tropics is largely a myth if one follows reasonable rules of health and sanitation.

REFERENCES

1. Wohl, M. G.: Dietotherapy, Clinical Application of Modern Nutrition, Philadelphia, 1945, W. B. Saunders Company, p. 797.
2. Keys, A., Taylor, H. L., Mickelsen, O., Henschel, A.: Famine Edema and the Mechanism of Its Formation, Science **103**: 669, 1946.
3. Morgan, H. J., Wright, I. S., van Ravenswaay, A.: Health of Repatriated Prisoners of War From the Far East, J. A. M. A. **130**: 995, 1946.
4. Official Records of Santo Tomas Internment Camp. Unpublished.

THE COMPARATIVE EFFICIENCY OF VARIOUS LIVER FUNCTION TESTS IN DETECTING HEPATIC DAMAGE PRODUCED IN DOGS BY XYLIDINE

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STUDIES concerning the comparative values of different liver function tests on human beings,[1-5] cats and rabbits,[6] and dogs,[7] with references to the original methods, have been published during the past few years. Hepatic damage in the cats and rabbits cited was induced by daily ingestion of selenium (from 0.1 to 1.0 mg. per kilogram) and, in the dogs, by small doses of carbon tetrachloride several times a week.

During the course of an investigation carried out in this laboratory with various species of animals, it was found that jaundice and fatty livers were produced in cats and dogs by a moderately low concentration of xylidine, an organic amine having the formula $C_6H_3(CH_3)_2NH_2$. This report is concerned with the evaluation of various liver function tests in reference to their efficiency and practicability in permitting early diagnosis in cases of experimentally induced liver damage in dogs.

EXPERIMENTAL METHODS

Thirteen adult female dogs weighing from 5.1 to 8.0 kilograms were used as experimental animals. They had been dewormed, kept under observation for at least six weeks prior to the experimental period, and had shown no signs of distemper or other respiratory infection.

Seven dogs (Group I), of which three were used as controls, were fed a diet (Diet A) consisting of 46 per cent horsemeat, 46 per cent kibbled biscuits, 4 per cent Sure's salt mixture,[8] and 4 per cent dried brewers' yeast. For comparative purposes, six dogs (Group II), of which three were used as controls, were fed a diet (Diet B) consisting of 30 per cent technical casein, 27 per cent crackermeal, 23 per cent sugar, 9 per cent Crisco, 4 per cent Sure's salt mixture, 4 per cent dried brewers' yeast, and 3 per cent cod-liver oil. The dogs in each group received 20 Gm. per kilogram of the assigned diets daily and showed a weight gain of approximately 0.1 kilogram weekly as long as they ate and retained the food. As will be noted, the results of the liver function tests and microscopic examination of the livers did not indicate any difference with respect to these diets.

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Hepatic damage was produced by exposing the dogs to a vapor-air mixture of xylidine of a high degree of purity. The inhalation exposures were of approximately six hours' duration daily, Monday through Friday. The average calculated concentration of xylidine in the chambers was 0.42 mg. per liter (85 p. p. m.), while the average concentration as determined by ultraviolet absorption was 0.22 mg. per liter (45 p. p. m.). Vomiting, loss of appetite, weakness, labored respiration, and jaundice were the usual symptoms observed. Weekly hematologic examinations revealed no marked changes. The survival time for the majority of the exposed dogs was approximately four or five weeks. Death occurred within a week after definite signs of jaundice were noted (jaundice occurring about one or two weeks after loss of appetite). On refusal of the dogs to eat the diet, forced feeding was instituted. Only one dog in Group II was able to retain the food. No signs of jaundice were noted even after fifteen weeks of exposure, when it was sacrificed and the liver found to be grossly nodular. Post-mortem examination of all the animals which died within five weeks showed marked jaundice around the limbs as well as the abdominal viscera, and the livers appeared pale and fatty.

In general, the methods of Smith and associates[6] for determining hepatic damage in cats and rabbits were used for the bromsulfalein, rose bengal, and bilirubin retention tests. In all these experiments, 5 mg. of the dye (or bilirubin) per kilogram body weight were injected intravenously. Examinations of the dye in the plasma were made at five- and thirty-minute intervals for the bromsulfalein and rose bengal retention tests and at 5- and 120-minute intervals for the bilirubin retention test. All the estimations were made with the Fisher spectrophotometer using appropriate filters and control plasma solutions. The amount of intravenously injected substance per cubic centimeter of plasma obtained at the different time intervals was read from a calibration curve. The per cent retention of the dye was expressed in terms of the amount found at thirty minutes (the per cent retention of bilirubin being based on the amount found at the end of 120 minutes) as compared to that found at five minutes. Although some of the injected dye or bilirubin is removed from the blood stream within the five-minute period, the per cent retention determined by the foregoing method tends to minimize the effect of variables, such as the total blood volume compared to the body weight, the hematocrit values, or possible inaccuracies in the administration of the dye or bilirubin.

The method of Bodansky[9] was followed in determining the serum phosphatase activity. Urobilinogen in the twenty-four hour urine specimens was determined by the method of Sparkman[10] following treatment of a sample of urine with freshly prepared ferrous hydroxide to reduce any urobilin as suggested by Schwartz and co-workers.[11] Values for icterus index were obtained by the visual comparison of saline-diluted plasma with potassium dichromate standards as outlined by Kolmer and Boerner.[12] The prothrombin clotting time was determined in plasma by the method of Quick[13] using freshly prepared thromboplastin made from rabbit brain. The prothrombin time ratio was expressed as the clotting time of the plasma of the exposed as compared to that of

the control animals. The direct and indirect van den Bergh tests were performed according to standard procedures.[12] The albumin-globulin ratio and fibrinogen estimations were carried out as outlined by Reiner.[14] Preliminary work with the cephalin-cholesterol test confirmed the observation of Hanger[15]

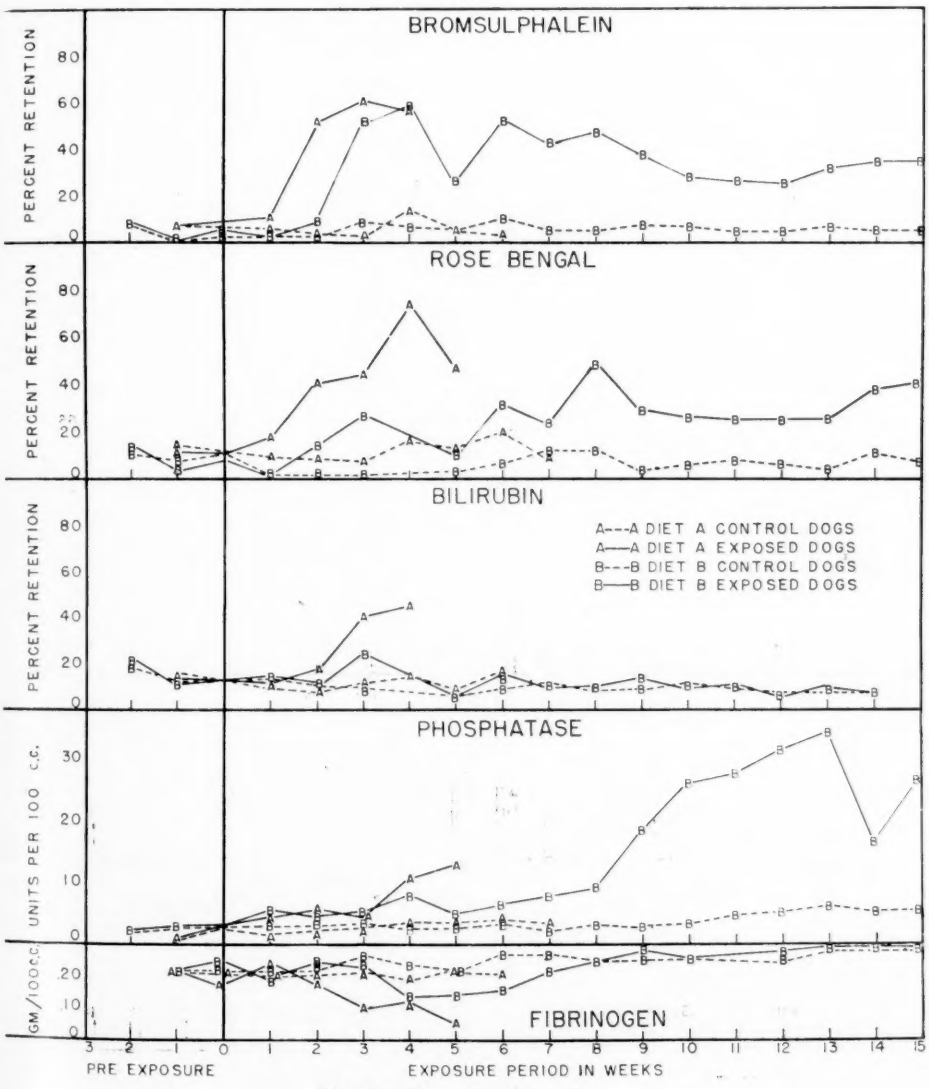

Fig. 1.—Liver function tests.

that marked flocculation occurred in the serum of normal dogs. For laboratory animals, this test has been reported to be of value only in monkeys.[16]

The various tests were performed at least sixteen hours after the previous day's exposure and feeding. All tests previously described were performed once a week, except the icterus index, prothrombin time ratio, and indirect van

den Bergh reactions which were determined twice weekly immediately after exposure. The urinalyses were done on twenty-four hour specimens collected each morning, Tuesday through Saturday.

Microscopic examination of the liver was done after fixation in a 4 per cent formaldehyde solution buffered to pH 7. Paraffin sections were routinely stained

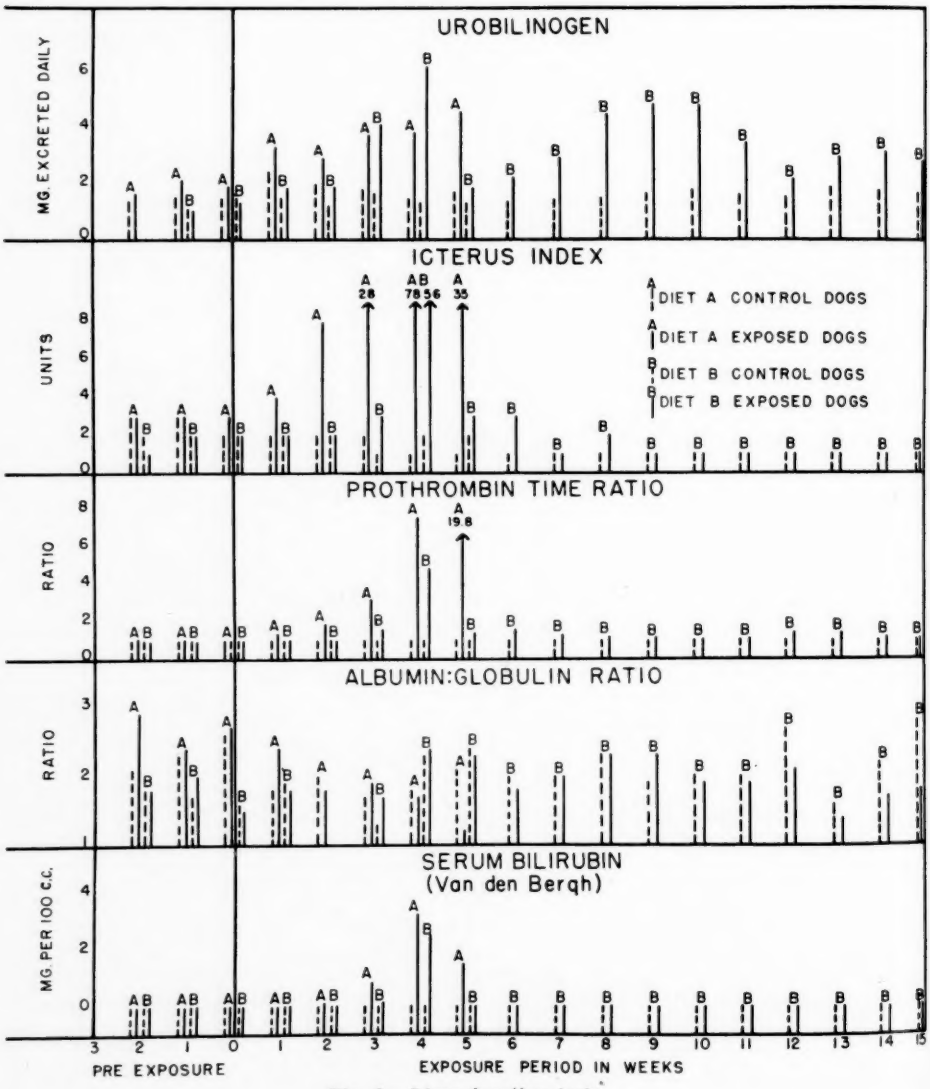

Fig. 2.—Liver function tests.

with hematoxylin-azure eosinate,[17] the modified van Gieson stain for hemoglobin,[18] and the acidulated ferrocyanide reaction for iron. Frozen sections were stained for fat according to the technique of Lillie and Ashburn.[19] Other special stains such as Mallory's aniline blue collagen stain[20] and Laidlaw's silver stain for reticulum[20] were used as desired.

Table I. Results of Liver Function Tests on Dogs of Group I (Diet A)

Weeks on test	Bromsulfalein retention (per cent)	Rose bengal retention (per cent)	Bilirubin retention (per cent)	Serum phosphatase (units per 100 c.c.)	Prothrombin time ratio	Urinary urobilinogen (mg. per day)	Comments
			Controls (Average of Dogs 12, 13, and 16)				
Pre-exposure	6.2	14.2	14.2	1.4	1.0	1.5	
1	4.9	8.7	8.9	2.3	1.0	2.4	
2	3.2	8.0	7.9	2.3	1.0	2.0	
3	2.6	7.6	10.7	2.6	1.0	1.8	
4	13.2	16.2	14.1	3.1	1.0	1.6	
5	4.6	12.9	8.6	2.8	1.0	1.7	
6	2.5	19.5	15.4	3.8	1.0	1.8	
7	–	8.6	–	3.2	1.0	2.1	Normal livers
			Dog 11				
Pre-exposure	5.7	12.3	15.5	1.4	1.1	1.9	
1	13.2	24.0	9.8	4.1	1.4	3.5	
2	62.0	44.5	20.0	3.8	2.2	3.6	
3	65.0	56.8	6.8	4.7	4.4	3.9	Jaundice noted
4	–	–	–		8.2	4.8	Precirrhotic liver
			Dog 14				
Pre-exposure	4.1	8.3	16.5	1.9	1.0	1.4	
1	13.6	19.5	22.7	6.3	1.2	3.7	
2	53.4	44.6	16.0	7.3	1.7	3.6	
3	66.7	49.5	84.0	6.1	3.2	4.8	Jaundice noted
4	–	–	–	16.6	10.7	2.1	Precirrhotic liver
			Dog 15				
Pre-exposure	4.8	4.7	13.1	1.5	1.0	2.0	
1	14.1	13.5	13.6	2.7	1.1	3.7	Jaundiced at death
2	–	–	–	5.2	1.6	2.4	Fatty metamorphosis of liver
			Dog 19				
Pre-exposure	0.0	10.4	4.0	1.5	1.0	2.5	
1	9.1	13.3	0.0	5.1	1.4	2.6	
2	39.0	31.8	14.6	5.1	1.8	2.2	
3	50.0	30.2	28.6	3.5	1.9	2.5	
4	56.0	73.0	44.5	4.7	3.6	4.3	Jaundice noted
5	–	46.2	–	12.7	15.3	4.6	Precirrhotic liver

TABLE II. RESULTS OF LIVER FUNCTION TESTS ON DOGS OF GROUP II (DIET B)

WEEKS ON TEST	BROMSULFALEIN RETENTION (PER CENT)	ROSE BENGAL RETENTION (PER CENT)	BILIRUBIN RETENTION (PER CENT)	SERUM PHOSPHATASE (UNITS PER 100 C.C.)	PROTHROMBIN TIME RATIO	URINARY UROBILINOGEN (MG. PER DAY)	COMMENTS
			Controls (Average of Dogs 21, 24, and 25)				
Pre-exposure	4.0	11.3	12.7	2.3	1.0	1.5	
1	2.4	0.0	14.5	2.9	1.0	1.5	
2	2.5	0.9	10.3	2.7	1.0	1.2	
3	7.5	1.3	11.6	3.0	1.0	1.7	
4	5.7	-	-	2.6	1.0	1.3	
5	4.8	3.2	6.7	2.6	1.0	1.3	
6	9.2	6.2	9.1	3.4	1.0	1.3	
7	4.2	9.5	11.3	2.0	1.0	1.4	
8	3.7	11.3	7.1	2.9	1.0	1.5	
9	6.4	3.4	8.3	2.8	1.0	1.6	
10	5.6	5.4	10.5	3.1	1.0	1.8	
11	3.9	7.3	8.1	4.5	1.0	1.6	
12	3.6	5.7	6.3	5.0	1.0	1.6	
13	5.5	3.5	6.0	6.1	1.0	1.9	
14	4.0	9.9	5.6	5.3	1.0	1.8	
15	3.4	6.4	-	5.7	1.0	1.7	Normal livers
				Dog 20			
Pre-exposure	2.4	6.9	10.6	1.7	1.0	1.4	
1	3.3	0.0	16.7	3.5	1.1	2.0	
2	8.4	27.3	9.1	2.8	1.0	1.8	
3	64.4	34.7	24.3	4.1	1.9	3.8	
4	89.0	-	-	8.2	9.4	10.4	Jaundice noted Precirrhotic liver

				Dog 23			
Pre-exposure	0.8	6.7	14.6	2.4	0.9	1.4	Jaundice noted
1	1.9	0.0	5.8	5.0	1.1	2.0	
2	5.7	8.4	12.7	4.6	0.9	2.0	
3	80.0	39.4	25.6	4.4	1.8	6.2	
4	66.0	-	-	10.7	33.8	6.4	Precirrhotic liver
				Dog 22			
Pre-exposure	2.7	10.0	9.2	4.3	1.0	1.2	
1	1.9	0.0	14.7	5.7	1.0	1.7	
2	7.9	6.2	9.2	5.2	1.0	2.1	
3	9.8	5.2	22.8	4.8	1.2	2.5	
4	16.2	-	-	4.4	1.3	2.1	
5	24.6	9.0	5.9	4.7	1.4	1.8	
6	51.2	31.2	15.7	6.6	1.6	2.3	
7	41.8	23.2	8.7	7.6	1.3	2.9	
8	45.7	48.0	8.7	9.3	1.2	4.5	
9	37.0	28.7	13.0	18.6	1.2	4.9	
10	26.8	25.7	8.8	25.4	1.1	4.7	
11	25.0	24.4	9.1	27.6	1.1	3.5	
12	23.7	24.6	4.6	31.0	1.4	2.2	
13	31.3	25.8	9.3	34.2	1.4	3.0	
14	33.2	37.0	6.9	17.6	1.2	3.1	
15	34.0	39.8	-	25.9	1.1	2.9	Cirrhotic liver with no signs of jaundice

RESULTS AND DISCUSSION

The data obtained from the tests are shown in Figs. 1 and 2 in which each value represents the average for all dogs surviving at that time.

The results of the liver function tests which gave early evidence of hepatic damage are shown in Tables I and II. Since the tests were performed on definite days of the week, some of the values are missing during the last week of exposure, due to the death or sacrifice of the animal.

Bromsulfalein Retention Test.—This test was the most sensitive and consistent of those studied. With the procedure followed in this experiment, it was concluded that, generally, retentions up to 10 per cent may be expected in normal dogs. Retentions from 10 to 15 per cent are suggestive of liver damage, and those above 15 per cent are definitely abnormal. All of the exposed dogs fed Diet A showed high retention of the dye by the end of the second week, three of the animals (Dogs 11, 14, and 15) having been in the suggestive range the previous week. Abnormal retention in Dogs 20 and 23, fed Diet B, occurred in the third week and in Dog 22 during the fourth week of exposure. The marked retention of the dye was noted soon after the dogs could not retain the food. For Dog 22, which retained its food, the abnormally high retention values reached the peak at the end of the sixth week and never exceeded the high values found in the rest of the dogs.

Rose Bengal Retention Test.—This test closely paralleled the bromsulfalein test. However, the values obtained on the control dogs varied more widely due to the difficulties in measuring dye concentrations in the presence of varying degrees of turbidity. It was found necessary to take initial blood samples for each dog prior to injection of the dye, the plasma being used as the blank in the spectrophotometer. Retentions up to 15 per cent were considered normal; 15 to 20 per cent, suggestive; and above 20 per cent, definitely indicative of liver damage. Ivy and Roth[21] have likewise reported that the rose bengal test in the dog gave comparable results to those obtained by the bromsulfalein test.

Bilirubin Retention Test.—This test was less sensitive and more erratic than either of the dye tests. Generally, abnormal retentions (those above 15 per cent) were not observed until one week after the dye tests had indicated liver injury. Due to the rapid excretion of bilirubin by dogs, the per cent retention seldom reached high levels even in cases of severely damaged livers. Drill and Ivy[7] concluded that this test was of little value in detecting liver damage in dogs poisoned by carbon tetrachloride. Smith and associates[6] have reported, however, an abnormal retention of bilirubin and of rose bengal in most of their chronically selenium-poisoned cats and rabbits.

Serum Phosphatase.—This test was found to be useful in detecting liver injury. A moderate rise in phosphatase activity (values above 4 units per 100 c.c. of serum being considered abnormal) was generally noted for all the dogs during the first week of exposure. High elevations were not noted until the fourth week of exposure. In the absence of normal values for individual subjects, this test would not appear to be as conclusive in making an early diagnosis of liver damage as either the bromsulfalein or rose bengal retention tests.

In the case of Dog 22, on Diet B, the moderate elevation of serum phosphatase, noted for the first eight weeks, rose to a markedly high level during the rest of the exposure period. In this latter period, the other tests either returned to the normal or had decreased values.

Prothrombin Time Ratio.—The rise in the prothrombin time ratio (values above 1.2 being considered abnormal) generally paralleled the increasing retention of bromsulfalein, except in the case of Dog 22. However, without supporting evidence, the increases noted in the ratio during the first three weeks were not sufficient to be conclusive. A marked increase in this ratio was usually noted prior to the death of the animal, when liver damage was indicated by all the other tests. This test is technically more difficult than the bromsulfalein retention test. Wide variations in clotting time may occur unless the amount of oxalate added to the blood is carefully controlled.

Urinary Urobilinogen.—This test did not appear to be as sensitive or as reliable as the bromsulfalein retention test. Values exceeding the excretion of 3 mg. per day of urobilinogen were considered abnormal. Considerable variation existed among the dogs regarding the time required for excreting high amounts of urobilinogen. The data obtained indicated that spot analysis of the urine is not satisfactory for urobilinogen estimations. Excretion of urobilinogen for individual dogs reached high levels for one or two days and then returned to normal. Useful information with this test can only be obtained by daily analysis of twenty-four hour samples of urine.

Van den Bergh Reaction.—This test was of little value in detecting early signs of liver damage, the direct and indirect reaction being obtained only when the dogs were jaundiced and near death. Bilirubin is not found in the blood of normal dogs, so appreciable amounts in the blood can only be expected in cases of severe liver damage.

Icterus Index.—In Group I, the icterus index rose above the normal values early in the exposure period but did not reach high values until death was near. Results in Group II were less conclusive, and the test was considered inferior to some of the others used.

Fibrinogen and Albumin:Globulin Ratio.—Neither of these tests showed changes which proved to be statistically significant. However, there was generally a decrease in fibrinogen prior to death of the animal.

PATHOLOGIC EXAMINATION

Dog 15, dying after ten days, showed marked fatty metamorphosis in the central two-thirds of the hepatic lobules.

The livers of Dogs 11, 14, 19, 20, and 23, dying within four to seven weeks, were precirrhotic. This was characterized by patchy and periportal proliferation of small basophilic spindle cells forming poorly marginated bands extending between adjacent portal areas and to the capsule. These cells formed strands or small tubules which at times closely resembled bile ducts. In addition, these bands had a few collagen and silver-positive reticulum fibers and were infiltrated by cells, chiefly lymphocytes.

Scattered through the parenchyma, often near the interportal bands, there were groups of basophilic regenerating liver cells. An occasional cell in these groups showed partial cytoplasmic oxyphilia. More often there were plugs of inspissated bile in the canaliculi.

Elsewhere, the fat-laden liver cells were usually swollen, oxyphilic, and occasionally frankly necrotic. Hemosiderin, generally in small amounts, was present in scattered Kupffer cells and less often in hepatic cells.

The liver of Dog 22, killed after fifteen weeks, was grossly nodular and cirrhotic. This was characterized, microscopically, by coarse fibrous trabeculations, irregularly traversing the liver, distorting the lobular architecture, and segregating nodules of basophilic liver cells. These bands contained small bile ducts and were infiltrated by many cells, chiefly lymphocytes and lipochrome-laden macrophages. Occasionally, small groups of liver cells, some of which were necrotic, were included in these bands.

The livers of the control animals showed no significant changes.

The other changes to be noted in the dogs dying within four to seven weeks were the presence of a few bile casts in the renal tubules and hemosiderin in scattered littoral cells, especially in the splenic pulp. These changes were absent in the dog killed at fifteen weeks.

SUMMARY AND CONCLUSIONS

Xylidine at a moderately low concentration is an effective agent in producing jaundice and fatty livers in dogs. With prolonged exposure, the livers pass through a precirrhotic to a cirrhotic stage. Microscopic examination of the liver showed evidence of parenchymal damage with no evident impairment in the cells of the reticulo-endothelial system, since these retain their function of phagocytosis of iron-positive pigment.

The bromsulfalein retention test was found to be the most reliable one in detecting liver damage. The rose bengal retention test was almost as sensitive but had certain disadvantages which made it less desirable.

The serum phosphatase activity and prothrombin time ratio were of equal value but less conclusive than the dye retention tests.

The bilirubin retention and urinary urobilinogen excretion tests gave indications of liver injury but were less consistent than the dye retention, serum phosphatase, or prothrombin time ratio tests.

Icterus index determinations may be of some value whereas the van den Bergh, fibrinogen, and albumin:globulin ratio estimations do not appear to yield information early enough to aid in the diagnosis of liver injury.

This order of efficiency agrees very well with the results obtained by Neefe and associates[5] in their work on liver damage produced in human subjects by administration of yellow fever vaccine and with the findings of Drill and Ivy[7] in carbon tetrachloride poisoned dogs.

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REFERENCES

1. Mateer, J. G., Baltz, J. I., Marion, D. F., Hollands, R. A., and Yagle, E. M.: A Comparative Evaluation of the Newer Liver Function Tests, Am. J. Digest. Dis. **9**: 13, 1942.
2. Mateer, J. G., Baltz, J. I., Marion, D. F., and MacMillan, J. M.: Liver Function Tests, J. A. M. A. **121**: 723, 737, 1943.
3. White, F. W., Deutsch, E., and Maddock, S.: The Comparative Value of Serial Hippuric Acid Excretion, Total Cholesterol, Cholesterol Ester and Phospholipide Tests in Diseases of the Liver. II. A Clinical Comparison of the Tests, Am. J. Digest. Dis. **7**: 3, 1940.
4. Rosenberg, D. H., and Soskin, S.: The Azorubin S. Test of Liver Function; an Evaluation With a Comparative Study of the Bromosulfothalein and Hippuric Acid Tests, Ann. Int. Med. **13**: 1644, 1940.
5. Neefe, J. R., Stokes, J., Jr., Reinhold, J. G., and Lukens, F. D. W.: Hepatitis Due to the Injection of Homologous Blood Products in Human Volunteers, J. Clin. Invest. **23**: 836, 1944.
6. Smith, M. I., Lillie, R. D., Stohlman, E. F., and Westfall, B. B.: Studies in Chronic Selenosis, Nat. Inst. Health Bull. No. 174, pp. 21-44, 1940.
7. Drill, V. A., and Ivy, A. C.: Comparative Value of Bromsulphalein, Serum Phosphatase, Prothrombin Time, and Intravenous Galactose Tolerance Tests in Detecting Hepatic Damage Produced by Carbon Tetrachloride, J. Clin. Invest. **23**: 209, 1944.
8. Sure, B.: Dietary Requirements for Fertility and Lactation; Existence of New Dietary Factor Essential for Lactation, J. Nutrition **22**: 499, 1941.
9. Bodansky, A.: Notes on Determination of Serum Inorganic Phosphate and Serum Phosphatase Activity, Am. J. Clin. Path. (Tech. Supp.) **7**: 51, 1937.
10. Sparkman, R.: Studies of Urobilinogin. I. A Simple and Rapid Method of Quantitative Determination of Urobilinogin in Stool and in Urine, Arch. Int. Med. **63**: 858, 1939.
11. Schwartz, S., Sborov, V., and Watson, C. J.: Studies of Urobilinogin. IV. The Quantitative Determination of Urobilinogin by Means of the Evelyn Photoelectric Colorimeter, Am. J. Clin. Path. **14**: 598, 1944.
12. Kolmer, J. A., and Boerner, F.: Approved Laboratory Technique, ed. 4, New York, 1945, D. Appleton-Century Company, Inc.
13. Quick, A. J.: The Clinical Application of the Hippuric Acid and the Prothrombin Tests, Am. J. Clin. Path. **10**: 222, 1940.
14. Reiner, M.: Manual of Clinical Chemistry, New York, 1941, Interscience Publishers, Inc.
15. Hanger, F. M.: Flocculation of Cephalin-Cholesterol Emulsions by Pathological Sera. Tr. A. Am. Physicians **53**: 148, 1938.
16. Kabat, E. A., Hanger, F. M., Moore, D. H., and Landow, H.: The Relation of Cephalin Flocculation and Colloidal Gold Reactions to the Serum Proteins, J. Clin. Invest. **22**: 563, 1943.
17. Lillie, R. D.: Romanowsky Staining With Buffered Solutions; Extension of Method to Romanowsky Stains in General, Stain Technol. **16**: 1, 1941.
18. Dunn, R. C., and Thompson, E. C.: New Hemoglobin Stain for Histologic Use; Slightly Modified Van Gieson Stain, Arch. Path. **39**: 49, 1945.
19. Lillie, R. D., and Ashburn, L. L.: Supersaturated Solutions of Fat Stains in Dilute Isopropanol for Demonstration of Acute Fatty Degenerations Not Shown by Herxheimer Technic, Arch. Path. **36**: 432, 1943.
20. Mallory, F. B.: Pathological Technique, Philadelphia, 1938, W. B. Saunders Company.
21. Ivy, A. C., and Roth, J. A.: The Limitations of Liver-Function Tests, Quart. Bull. Northwestern Univ. M. School **17**: 179, 1943.

STUDY OF ABSORPTION FROM CRYSTALLINE INSULIN PELLETS AND SOLUTIONS AT VARIOUS SITES IN RABBITS

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THE study herein reported was initiated primarily to determine if absorption from insulin pellets varied at different sites of implantation. Insulin pellet implants have been reported;[1-3] however, to our knowledge there is no information relative to insulin implants in the spleen. It was believed that study of splenic implants would be of interest inasmuch as the venous drainage from both the spleen and pancreas goes mainly to the liver via the portal system. Therefore, absorption from splenic implants would closely follow the route of normal endogenous insulin after its release by the pancreas. Any hepatic regulatory effect on insulin would be expected to play its part in absorption from splenic implants, whereas little or no effect from the liver would be expected in the case of intramuscular or subcutaneous pellets.

MATERIAL AND METHODS

Crystalline zinc insulin with a potency of 25.89 units per milligram was used throughout the experiments. Pellets were made by compressing the material as tightly as possible in a small mechanical hand press. The pellets had a diameter of approximately 2 mm. and a thickness of ½ to 1 mm. depending on the weight of insulin used. The rabbits used in the pellet implantation experiments were New Zealand Whites weighing from 3.5 to 4.5 kilograms. They were starved for a twenty-four hour period preceding the experiment and during the blood sampling period, but water was given.

Sodium pentothal was used as the anesthetic. Each rabbit was given an initial intravenous injection of 2 c.c. of 2 per cent solution fairly rapidly. This resulted in light anesthesia. Additional amounts of sodium pentothal solution were then given slowly until surgical anesthesia was reached. The rabbits tolerated pentothal very well. Only one rabbit out of forty-one was lost from respiratory failure and that was the third animal used in the series. The amount of 2 per cent sodium pentothal solution used varied from 2.5 to 10 c.c. with an average of 4.9 c.c. per rabbit.

The splenic implants were performed as follows. The upper abdomen was opened with a midline incision, and the spleen was brought to the surface. The arterial supply was clamped off lightly and a small incision made through the fibrous capsule. The pellet was then inserted deeply in the splenic pulp and the capsule closed with a single silk suture. The clamp was then removed. and the abdomen was closed with linen sutures. The skin was closed by metal clips. A bandage was wrapped around the abdomen to prevent trauma to the wound from the animal's claws.

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The intramuscular pellets were inserted in the substance of the trapezius muscle, and the muscle incision was closed with a silk suture. The skin incision was closed with metal clips. The subcutaneous pellets were implanted in the scapular area, and the skin incision was closed with metal clips. Controls were run with 5 c.c. of 2 per cent sodium pentothal solution to rule out any effect of the anesthetic on the blood sugar level. A second series of controls were run using starch pellets implanted in the spleen to rule out any effect of the operative procedure on the blood sugar. Blood samples were taken from the marginal vein of the ear just before the operation and at four, five, twenty-four, and thirty hours after the implant was made. Glucose determinations were made by the micro method of Hagedorn-Jensen.

A solution containing 50 units per cubic centimeter of the crystalline insulin was made up in slightly acidulated water containing 0.1 per cent phenol. A group of ten fasting rabbits were injected subcutaneously with 0.8 units each and a second group of ten with the same amount intramuscularly. Blood samples were taken before injection and at three-fourths, one and one-half, two and one-half, and four hours following injection. The groups were reversed a week later.

RESULTS AND DISCUSSION

The results of the pellet implants and anesthetic controls are shown in Table I and in Fig. 1.

The sodium pentothal solution given intravenously had very little effect on the blood sugar level. Blood sugar values at four and five hours were slightly but not significantly lower than before the anesthetic was given.

TABLE I

EXPERIMENTAL PROCEDURE	TOTAL NUMBER OF ANIMALS IN EXPERIMENT	DEATHS WITHIN 24 HOURS		DEATHS FROM 24 TO 48 HOURS		KILLED INSULIN SHOCK FROM 24 TO 48 HOURS		SURVIVAL AFTER 48 HOURS		PELLET SIZE (MILLIGRAMS)	
		NO.	(%)	NO.	(%)	NO.	(%)	NO.	(%)	VARIATION	AVERAGE
Insulin pellets											
Subcutaneous	10	8	80	0		1	10	1	10	2.9 to 2.1	2.56
Intramuscular	10	4	40	0		2	20	4	40	5.3 to 2.1	3.07
Intrasplenic	10	4	40	1	10	1	10	4	40	6.5 to 3.0	3.65
Starch pellets											
Intrasplenic	5	0		0				5	100	10.0 to 5.0	8.00
Pentothal											
Intravenous 5 c.c. 2 per cent	5	0		0				5	100		

The operation with starch implants intrasplenically resulted in a moderate increase in the blood sugar in 80 per cent of the animals four and five hours after the operation. All of the controls survived during the thirty-hour observation period. The subcutaneous insulin pellet implants produced a pronounced drop in the blood sugar to an average value of 49.9 mg. per cent glucose at four hours and 46.4 mg. per cent glucose at five hours after the operation. Eight out of ten rabbits died in insulin shock within the twenty-

four hour period following the implantation. Examination of the pellets after the death of the animals revealed that they had become very soft and, in several cases, had lost most of their shape.

The intramuscular insulin implants resulted in a decrease in blood sugar level to an average value of 60.6 mg. per cent glucose at four hours and 57.4 mg. per cent glucose at five hours after the implantation. Four out of ten rabbits died in insulin shock within twenty-four hours of the operation. Examination of the pellets, after the death of the rabbits, revealed that they retained their shape fairly well but were soft. The twenty-four and thirty-hour blood sugar levels on the six surviving animals were 23.4 and 11 mg. per cent lower than the initial value showing a definite hypoglycemic action for at least thirty hours.

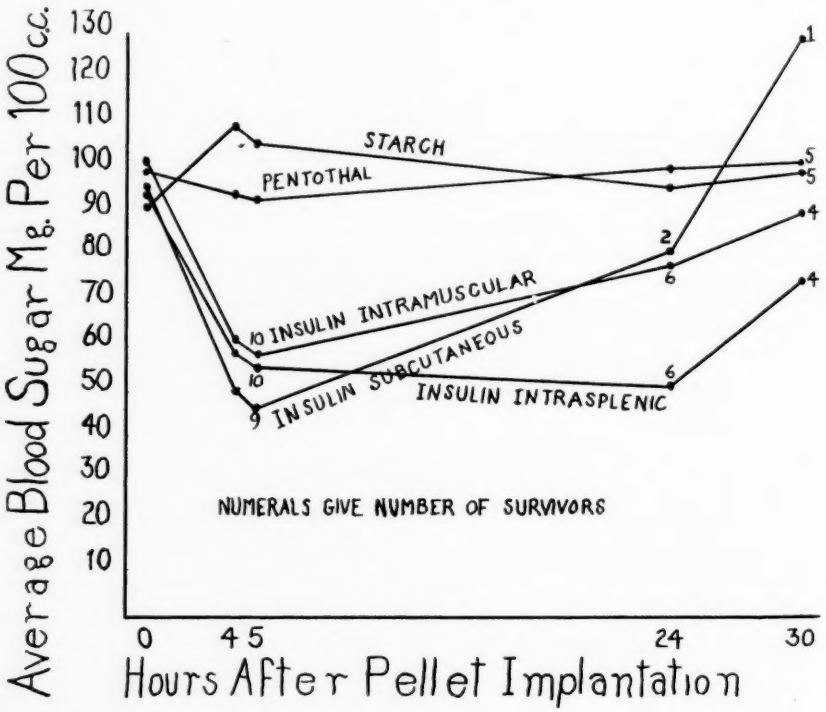

Fig. 1.

The intrasplenic insulin implants gave a hypoglycemic effect very similar to the intramuscular implants at the four- and five-hour periods, the blood sugars being 58.8 and 55.3 mg. per cent glucose, respectively. The twenty-four hour value (six rabbits) was 51 mg. per cent glucose, while the thirty-hour value (four rabbits) was almost 20 mg. per cent lower than the initial level. The splenic implants hence gave a somewhat longer insulin release than the intramuscular pellets. There was very little change in the shape of the pellets in the spleen although they became quite soft. There was no evidence of any regulatory action by the liver on the insulin released from splenic

TABLE II

EXPERIMENTAL PROCEDURE	BLOOD SUGAR (MILLIGRAMS PER CENT GLUCOSE)				
	INITIAL	¾ HOUR	1½ HOURS	2½ HOURS	4 HOURS
0.8 Units insulin subcutaneous	106.4	78.4	70.2	74.3	104.5
0.8 Units insulin intramuscular	103.7	76.3	72.3	84.9	109.9

implants. The results of the intramuscular and subcutaneous injections are shown in Table II. The two and one-half hour value with the intramuscular injection is somewhat higher than with the subcutaneous injection. There are, however, no differences between the two curves either in the rapidity of onset or depth of the hypoglycemia. This result would indicate that the more rapid absorption from subcutaneous insulin pellets is probably due to mechanical factors with a greater tendency for the subcutaneous pellet to be disrupted. This is in agreement with the appearance of the pellets after the deaths of the rabbits.

CONCLUSIONS

Subcutaneous insulin pellet implants in rabbits result in a more rapid and intense hypoglycemic action than intramuscular or intrasplenic implants. This is probably due to mechanical factors that cause a more rapid breakdown of the pellet.

Intrasplenic insulin pellet implants produce a more prolonged insulin action than intramuscular implants. There is apparently no appreciable hepatic regulation on the insulin released from the splenic implants.

There are no marked differences in the insulin action in rabbits between intramuscular and subcutaneous injections with regard to speed of onset and depth of hypoglycemia.

REFERENCES

1. Parkes, A. S., and Young, F. G.: Influence of Subcutaneous Implantation of Tablets of Solid Insulin on the Blood Sugar Level of the Rabbit, J. Endocrinol. **1**: 108, 1939.
2. Cutting, W. C., Morton, M. C., and Cohn, R. B.: Subcutaneous Administration of Insulin by Pellets, Endocrinology **28**: 679, 1941.
3. McCullagh, E. P., and Lewis, L. A.: Studies on the Effectiveness of Insulin Implants, Cleveland Clin. Quart. **9**: 153, 1942.

THE NATURE OF THE ACTION OF DIMETHYLAMINOETHYL BENZHYDRYL ETHER HYDROCHLORIDE (BENADRYL): EFFECTS UPON THE HUMAN EYE

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THE antihistamine action of Benadryl has been well established,[1-19] and upon this much of its weaker antispasmodic effect may secondarily depend.[3, 8, 20, 21] Because of its antagonism to the depressor and spasmogenic influence of acetylcholine, an atropine-like activity has been claimed.[1, 3, 10, 16, 22] The dryness of the mouth sometimes seen clinically may be a manifestation of this character. On the other hand, others have observed an increase in the pressor action of epinephrine,[22] although this has not been confirmed at the bedside.[16] Symptoms and signs of sympathomimetic action are conspicuously absent in patients who have taken the drug in quantities up to 600 mg. daily.[16] No increase in pulse rate or blood pressure is evident; dryness of the mouth is rare; and palpitation has not been noted. "Jitteriness" and "inward nervousness" are not only absent, but placidity and even drowsiness may occur;[16] when the drug is given orally or intravenously, it causes no dilatation of the pupil of the eye.[16] Effort has been made in the present study to extend the observations in connection with the oral administration of the drug and to record the effects of the alkamine ether following its topical application to the eye, thereby clarifying certain points regarding the fundamental nature of its action.

METHODS AND RESULTS

The Effect of Orally Administered Benadryl.—The pupils of twenty ambulatory subjects, without known disease of the autonomic nervous system or organic disease of the eye, were observed before, periodically during, and after the use of benadryl orally in daily doses of 150 to 400 mg. for periods of time ranging from three to twelve weeks. Throughout the period of study, a constant diet was used, other drugs were not employed, and normal freedom of the hospital wards was allowed. Measurements of pupillary diameters with an especially adapted millimeter ruler were carried out under constant conditions of lighting, while the attention of the patient was directed to an object, the character and position of which did not vary.

No perceptible change in pupillary size nor in the ability of the subject to read a Snellen chart were observed in any one of these subjects as a result of the administration of benadryl.

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The Effect of Topically Applied Benadryl.—For these experiments, sixty ambulatory subjects were chosen at random, save for the exclusion of anyone with an organic disease of the eye or a clinically recognizable disturbance of the autonomic nervous system. The ages of these individuals ranged from 21 to 65 years. All applications were made to the right eye; the "fore" reading and readings made on the left eye all served as controls. At no time in any one experiment was the size or accommodative capacity of the left eye perceptibly affected. One drop of a fresh 0.5 per cent aqueous solution was instilled at ten-second intervals for three doses, unless otherwise noted. All readings were taken at 0-, 15-, and 60-minute intervals, respectively, in relation to the instillation of the drug.

The Influence of Benadryl Alone: A definite increase in the size of the pupil was observed in forty-eight of the sixty subjects, averaging approximately 1.9 mm. at the end of one hour (Table I). In the remaining twelve, no change was observed; the washing out effect of the excessive flow of tears may have played a part in this failure to react.

As a rule, a maximum dilatation was present by the end of an hour and had begun to decrease quite noticeably within two hours. In only four patients was any dilatation still measurable at the end of twenty-four hours.

TABLE I. CHANGES IN VISUAL APPARATUS OF SIXTY SUBJECTS FOLLOWING TOPICAL APPLICATION OF BENADRYL (0.5 PER CENT AQUEOUS SOLUTION)

	INCREASED IN	DECREASED IN	UNCHANGED IN
Pupillary size	48	0	12
Visual acuity	0	12	48
Accommodation	0	43	17

Visual acuity, as determined by the Snellen chart read at a 20-foot distance, was decreased in twelve subjects and unaltered in forty-eight (Table I). No attempt was made to correlate these findings with the presence or absence of astigmatism. Capacity for accommodation was tested separately for each eye before and sixty minutes after the instillation of benadryl with the aid of a Prinz accommodation ruler and a Jaeger reader No. 3. In each instance, the chart was moved toward the subject at a uniform speed from an easy reading distance. The point at which the chart became blurred and was no longer readable was accepted as the "limit of accommodation." Both before and after benadryl, the results of from three to six readings were averaged to obtain the final figure recorded. Patients unable to give reliable results prior to the instillation of benadryl were eliminated from the experiment. Forty-three of the sixty patients showed a decreased capacity for accommodation. The average "limits of accommodation" for these forty-three subjects before and after benadryl were 10.3 and 13.1 cm., respectively. Corresponding figures determined for the entire group of subjects were 10.7 and 12.7 cm., respectively.

The Effect of Epinephrine Upon the Action of Benadryl: In these and subsequent experiments, three drops of benadryl were used in the right eye, whereas only one drop of each of the other solutions, respectively, was em-

ployed. Whenever a second agent was tested (unless otherwise stated), it was given first, and readings were made at 0, 15, and 60 minutes, respectively. Immediately after the sixty-minute period, the benadryl solution was added with additional observations at 75 and 120 minutes, respectively.

The results of these and all subsequent experiments on the size of the pupil, for which fifteen subjects were used, are summarized in Table II. In all instances, the size of the pupils in the "fore" period served as a control, and the diameter found has been expressed arbitrarily as 100. The "after" readings have been recorded as a percentage figure of this "normal." In six patients benadryl and epinephrine (0.001 per cent) were instilled, one immediately after the other, to a total of three drops for each. The total ensuing dilatation was no greater than with benadryl alone but reached its maximum earlier, that is, in fifteen minutes rather than in one hour.

The mydriasis following a single drop of epinephrine was slight in degree and did not influence the response to subsequently instilled benadryl (Table II).

The Effect of Homatropine Upon the Action of Benadryl: One drop of a 1.0 per cent aqueous solution of homatropine sulfate caused an average increase of 156 per cent in the size of the pupil of the right eye of each of fifteen subjects. The subsequent administration of benadryl caused a small additional average widening (169 per cent) (Table II), although two of the fifteen patients did not respond at all.

TABLE II. COMPARISON OF EFFECTS UPON PUPILLARY SIZE OF BENADRYL AND OTHER AGENTS, SINGLY AND IN COMBINATION

SUBJECT NO.	BENADRYL (0.5%)	EPINEPHRINE HCl (0.001%)	EPINEPHRINE HCl PLUS BENADRYL	HOMATROPINE SO_4 (1.0%)	HOMATROPINE SO_4 PLUS BENADRYL	ESERINE SO_4 (0.5%)	ESERINE SO_4 PLUS BENADRYL
1	100	100	100	200	200	25	22
2	108	108	100	133	140	33	33
3	160	102	140	175	200	17	40
4	150	100	133	150	175	40	48
5	100	100	100	150	150	38	38
6	138	110	140	120	150	67	67
7	127	100	129	167	187	25	55
8	150	113	125	175	195	40	63
9	120	100	140	160	165	33	42
10	125	100	133	140	147	50	55
11	120	100	133	167	177	33	33
12	140	108	138	160	166	33	50
13	124	104	122	180	192	57	67
14	120	105	120	145	163	28	44
15	123	102	119	128	130	51	56
Average	127.0	104.1	124.8	156 6	169.1	38.0	47.6

The Effect of Eserine Upon the Action of Benadryl: Eserine sulfate, preferably termed physostigmine sulfate (0.5 per cent) produced a characteristic miosis in which the average diameter of the pupillary aperture was reduced to 38.0 per cent of its former size. In five of fifteen patients, the subsequent application of benadryl, under the conditions of the experiment, had no influence upon the pupillary opening, but the average increase in diameter for the entire group was of sufficient magnitude to augment the size of the pupil from 38.0 to 47.6 per cent of its initial value (Table II).

CONCLUSION

While certain variations of the previously mentioned procedures might permit more accurate measurements of the effects of benadryl upon the eye, the results of the dosage, time of application, frequency of observation, and methods of measurement arbitrarily employed have afforded data capable of clear interpretation. The partial paralysis of accommodation, the slight inhibition of the miosis caused by eserine, and the furthering effect of benadryl upon the mydriasis produced by epinephrine and atropine all seem to point toward an atropine-like or vagal-paralyzant effect of benadryl when topically applied to the eye.

SUMMARY

1. Orally administered dimethylaminoethyl benzhydryl ether hydrochloride (benadryl) in doses up to 400 mg. daily does not influence the size of the pupil of the eye.

2. Benadryl topically applied to the bulbar conjunctiva in 0.5 per cent aqueous solution causes within fifteen minutes a moderate mydriasis of readily measurable proportions; this becomes maximal within one hour. The capacity for accommodation is simultaneously decreased.

3. Epinephrine accelerates the action of benadryl upon the pupil but under the conditions of these experiments does not increase its intensity.

4. Benadryl furthers the mydriatic effect of homatropine and lessens the miosis produced by eserine.

5. The probable nature of the ocular action of benadryl is mentioned.

REFERENCES

1. Code, C. F.: A Discussion of Benadryl as an Antihistamine Substance, Proc. Staff Meet., Mayo Clin. **23**: 439, 1945.
2. Curtis, A. C., and Owens, B. B.: Beta Dimethylaminoethyl Benzhydryl Ether Hydrochloride (Benadryl) in Treatment of Urticaria, Arch. Dermat. & Syph. **52**: 239, 1945.
3. Dufresne, R. R.: Les substances antihistaminiques, Union méd. du Canada **75**: 59, 1946.
4. Editorial: Benadryl—A New Antihistamine Substance, Canad. M. A. J. **54**: 59, 1946.
5. McGavack, T. H., and Elias, H.: Influence of Dimethylaminoethyl Benzhydryl Ether Hydrochloride Upon Histamine Flare Reactions, Proc. Soc. Exper. Biol. & Med. **61**: 133, 1946.
6. Loew, E. R., and Kaiser, M. E.: Alleviation of Anaphylactic Shock in Guinea Pigs With Synthetic Benzhydryl Alkamine Ethers, Proc. Soc. Exper. Biol. & Med. **58**: 235, 1945.
7. Loew, E. R., Kaiser, M. E., and Moore, V.: Synthetic Benzhydryl Alkamine Ethers Effective in Preventing Fatal Experimental Asthma in Guinea Pigs Exposed to Atomized Histamine, J. Pharmacol. & Exper. Therap. **83**: 120, 1945.
8. Loew, E. R., Kaiser, M. E., and Moore, V.: Effect of Various Drugs on Experimental Asthma Produced in Guinea Pigs by Exposure to Atomized Histamine, J. Pharmacol. & Exper. Therap. **86**: 1, 1946.
9. Loew, E. R., Kaiser, M. E., and Anderson, M.: The Antihistamine Action of Alkyloxytriazines and Related Compounds, J. Pharmacol. & Exper. Therap. **86**: 7, 1946.
10. Loew, E. R., Kaiser, M. E., and Anderson, M.: The Antihistamine and Atropine-like Properties of Quaternary Ammonium Derivatives of Benadryl, Federation Proc. **5**: 190, 1946.
11. McElin, T. W., and Horton, B. T.: Clinical Observations on the Use of Benadryl: A New Antihistamine Substance, Proc. Staff Meet., Mayo Clin. **23**: 417, 1945.
12. McGavack, T. H., Elias, H., and Boyd, L. J.: The Influence of Dimethylaminoethyl Benzhydryl Ether Hydrochloride Upon Gastric Acidity, Gastroenterology **6**: 439, 1946.

13. McGavack, T. H., Elias, H., and Boyd, L. J.: The Influence of Dimethylaminoethyl Benzhydryl Ether Hydrochloride (Benadryl) Upon Normal Persons and Upon Those Suffering From Disturbances of the Autonomic Nervous System, J. LAB. & CLIN MED. **31**: 560, 1946.
14. Page, I. H., and Green, A. A.: The Vascular Action of B-Dimethylaminoethyl Benzhydryl Ether Hydrochloride (Benadryl), Federation Proc. **5**: 78, 1946.
15. Selle, W. A.: Beta Dimethylaminoethyl Benzhydryl Ether Hydrochloride as an Antihistamine and Antianaphylactic Agent, Federation Proc. **5**: 93, 1946.
16. Sharp, E. A.: Personal communication.
17. Wells, J. A., and Morris, H. C.: Observations on the Antagonism of Histamine by B-Dimethylaminoethyl Benzhydryl Ether (Benadryl), Federation Proc. **4**: 140, 1945.
18. Wells, J. A., Morris, H. C., Bull, H. B., and Dragstedt, C. A.: Observations on the Nature of the Antagonism of Histamine by B-Dimethylaminoethyl Benzhydryl Ether (Benadryl), J. Pharmacol. & Exper. Therap. **85**: 122, 1945.
19. Wells, J. A., Morris, H. C., and Dragstedt, C. A.: Modification of Anaphylaxis by Benadryl, Proc. Soc. Exper. Biol. & Med. **61**: 104-6, 1946.
20. Ellis, F. W.: The Effects of Some New Spasmolytic Agents in Perfused Guinea-Pig Lungs, Federation Proc. **4**: 117, 1945.
21. Ellis, F. W., and Newsome, J. F.: The Effect of Certain New Antihistamine Drugs on Bronchial Spasm, Federation Proc. **5**: 176, 1945.
22. Sherrod, T. R., Schloemer, H. F., and Loew, E. R.: Pharmacological Studies of Antihistamine Compounds, Federation Proc. **5**: 202, 1945.

LABORATORY METHODS

CONSTANT FLOW GASSING CHAMBERS: PRINCIPLES INFLUENCING DESIGN AND OPERATION

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INTRODUCTION

ALTHOUGH dynamic (or constant flow) chambers for the experimental gassing of animals or human subjects have been described previously,[1-10] the basic principles underlying the operational behavior of such chambers have not been discussed. It is the purpose of this paper to present such a discussion. Experimental data for various gases are given in verification of the principles described.

DESCRIPTIVE

A dynamic chamber is one through which air is continuously drawn at a fixed rate. The agent to be investigated is introduced into the affluent air as a vapor, smoke, dust, or fog. Animals to be exposed are introduced into the chamber through a door, after the desired concentration has been established. The constant stream of air through the chamber renews the oxygen supply for the animals and removes the exhaled moisture and carbon dioxide. Concentrations may be maintained by this system with comparative ease.

However, it is essential that the agent be dispersed at a constant rate through the period of operation. The upper limit of concentration obtainable is fixed by the physical characteristics of the disseminated chemical and the method of dispersion. A discussion of methods or apparatus for the dispersion of agents in various states is not within the scope of the present report.

The flow of air through the chamber is maintained by a pump, usually in the effluent line, and is measured by an accurately calibrated orifice meter, rotameter, or similar device. To insure an even air flow, buffer tanks may be installed to eliminate the effect of pump pulsations. Since with the pump in the effluent line the pressure within the chamber is always slightly less than atmospheric, leakages outward from the chamber cannot occur. Thus, extremely toxic compounds can be biologically assayed without danger to the experimenter.

ESTIMATION OF CONCENTRATION

Concentrations of agents in the chamber may be either calculated theoretically or determined by analysis. The theoretical or "nominal" concentration, as it has been called in Chemical Warfare Service laboratories for many years, is calculated from the quantity of agent introduced into the chamber per minute and the rate of air flow through the chamber, as follows:

$$\text{Nominal concentration in milligrams per liter} = \frac{\text{agent flow in milligrams per minute}}{\text{air flow in liters per minute}}$$

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The validity of such nominal concentrations depends on the accuracy of measurement of both the rate of flow of the agent into the chamber and the rate of flow of air through the chamber, *always provided that both of these flows are constant.*

The analytical concentration is measured by chemical or physical analysis of a sample of air drawn from the chamber. If the collection of the sample is complete and the method of analysis accurate, the analytical concentration is the actual concentration within the chamber at the time of sampling. Lack of agreement between the analytical and nominal concentrations may be due to one or more of the factors discussed herein.

EQUILIBRATION

When a vapor or gas is introduced at a uniform rate into a chamber through which there is a continuous flow of air, the concentration within the chamber increases until it is practically constant. If it is assumed that perfect mixing with the chamber air occurs, the concentration at any time may be calculated by the following modification of the usual ventilation equation[11]:

$$C = \frac{w}{b}\left(1 - e^{\frac{-bt}{a}}\right) \qquad (1)$$

Where C = the concentration in milligrams per liter at time t
w = milligrams of agent introduced per minute
a = the volume of the chamber in liters
b = the volume of air passing through the chamber each minute.

The percentage of the desired concentration $\frac{w}{b}$ obtained in time t is then given by:

$$\text{Per cent} = 100\left(1 - e^{\frac{-bt}{a}}\right) \qquad (2)$$

From Equation 1, it is apparent that the chamber concentration, at first, rises rapidly and then approaches a constant value at infinite time. Consequently, for practical purposes, animals may be introduced into the chamber when the concentration reaches 99 per cent of the theoretically constant value, since the rise in concentration from that point is less than 1 per cent for any finite exposure period. The time required for equilibration of the chamber to 99 per cent may be calculated by setting Equation 2 equal to 99:

$$100\left(1 - e^{\frac{-bt}{a}}\right) = 99$$

or

$$e^{\frac{-bt}{a}} = \frac{100 - 99}{100} = 0.01$$

which, when transformed into logarithmic form, becomes:

$$-\frac{bt}{a} = \ln 0.01 = -4.6052$$

and

$$t_{99} = 4.6052\,\frac{a}{b} \qquad (3)$$

Thus, if the size of the chamber (a) and the rate of air flow (b) are known, the time for reaching 99 per cent of the theoretical concentration at infinite time can be estimated easily from Equation 3.

This equation may be given the general form:

$$t_x = K \frac{a}{b} \qquad (4)$$

where x = per cent of nominal concentration attained in time t, and K = a constant.

Values of K for various values of x are

x	K
99	4.605
95	2.996
90	2.303
85	1.897
80	1.609

Experimental verification of this principle is presented as follows.

In one series of experiments, chlorine was introduced at a constant rate into a 629 liter chamber, operated at three different constant air-flow levels, 125, 250, and 500 liters per minute. Samples were drawn at regular intervals and analyzed until three analyses coincided. In Table I a comparison is made between these analytical concentrations, expressed as percentages of the final

TABLE I. TIME TO REACH CONSTANT CONCENTRATION IN 629 LITER CHAMBER

CHAMBER AIR FLOW (LITERS PER MINUTE)	TIME (MINUTES)	PER CENT FINAL CONCENTRATION	
		OBSERVED	CALCULATED
125	2	32	33
	4	56	55
	6	70	70
	8	78	80
	10	84	86
	12	89	91
	14	90	94
	16	99.5	96
	18	100	97
	20	100	98
	22	100	99
250	2	56	55
	3	71	70
	4	80	80
	5	90	86
	6	90	91
	7	94	94
	8	97	96
	9	99	97
	10	99	98
	11	100	99
	12	100	99.2
	13	100	99.4
500	2	80	80
	3	92	91
	4	96	96
	5	99	98
	6	100	99.2
	7	100	99.6
	8	100	99.8

concentration, and the theoretical per cent of the constant value concentration, which should be reached at the time of sampling, as predicted by the use of Equation 2. The results, given in Table I, showed a close agreement between the observed and calculated values.

In another series of experiments, dichlorodiethyl sulfide was dispersed into a 20,000 liter chamber which was operated at an air flow of 3,600 liters per minute in Table II, the actual per cent of the final concentrations found by sampling at regular intervals are compared with calculated values, as in Table I. The observed and calculated percentages also showed close agreement.

TABLE II. TIME TO REACH CONSTANT CONCENTRATION IN 20,000 LITER CHAMBER; AIR FLOW, 3,600 LITERS PER MINUTE

TIME (MINUTES)	PER CENT FINAL CONCENTRATION	
	FOUND	CALCULATED
2	33	30
5	57	59
8	77	76
11	86	86
14	93	92
17	97	95
20	97	97
23	100	99

It may be seen from Equation 3 that t_{99} is independent of any previous concentration level in the chamber. Therefore, for any fixed chamber volume and air flow, the time required for a change from one state of equilibration to another is always the same. It is thus obvious that t_{99}, for clearing the chamber after stopping the flow of agent, is identical with the t_{99} for establishing the concentration. Moreover, if the concentration in the chamber is changed after equilibration, the time for attaining the new level also is the same as the equilibration time.

For low air flows and high sampling rates, the rate of sample collection should be added as a correction to the chamber air flow for the most accurate calculations of equilibration time. However, when the sampling rate is less than 1 per cent of the air flow, this correction may be considered negligible and can be omitted.

Ideally, after equilibration, the analytical concentration should approximate the nominal concentration. Actually, perhaps because of surface effects within the chamber, there may be a significant discrepancy.

SURFACE EFFECTS

The interior surface of the chamber to which the agent is exposed may lower its concentration because of absorption, adsorption, or chemical reaction. An illustration of surface effects is given by the following experimental data. Concentrations of chlorovinyl dichloroarsine were established in a 386 liter chamber operated at 250 liters per minute. After equilibration, samples were collected and analyzed by iodometric titration. In one series, the interior surfaces of the chamber were coated with a cellulose acetate lacquer. In another

series, the surfaces were covered with a "corrosion-proof" lacquer.* The results are given in Table III, which shows that the cellulose acetate coating caused a much greater decrease in concentration than did the "corrosion-proof" lacquer.

TABLE III. EFFECT OF TYPE OF SURFACE ON CHAMBER CONCENTRATION AGENT, CHLOROVINYL DICHLOROARSINE

SURFACE	NUMBER OF EXPERIMENTS	AVERAGE NOMINAL CONCENTRATION FOUND (PER CENT)	S.D.
Cellulose acetate	8	13	±9
Cotoid	39	93	±5

This effect may be minimized by selection of the proper surface for each agent to be used in the chamber. Thus, the interior may be glass- or enamel-lined or lacquered. For most compounds, a corrosion-resistant lacquer is sufficient to prevent appreciable loss of concentration. However, it should be emphasized that the presence of even small surfaces of rubber will decrease the concentrations of certain organic vapors to a marked degree. For example, a 1 foot section of ¼ inch rubber tubing in the sampling line completely absorbed the chlorovinyl dichlorarsine in a 10 liter sample containing 0.25 mg. per liter. The same sized section of rubber tubing thrown into a gassing chamber reduced the concentration of the same compound in the chamber by about 20 to 30 per cent. Similar effects of rubber have been noted with dichlorodiethyl sulfide and other organic sulfides. In this laboratory, all rubber surfaces in the chamber and sample collection line are either coated with lacquer or are eliminated by using either ground glass joints or glass to glass connections in assembling sampling lines. Inasmuch as the type and extent of interaction of surface upon any particular agent cannot be predicted, it is necessary to make analytical-nominal concentration comparisons to ascertain this effect before any experimental gassing is performed.

The magnitude of any surface effect depends on the quantity of surface exposed per unit volume and is governed by the shape and size of the chamber and the presence of extraneous interior surface. The surface/volume ratio is a function largely of the size and shape of the chamber. A spherical chamber has the smallest surface/volume ratio. However, a cubical chamber is more easily constructed, and since it has a lower surface/volume ratio than the other rectangular shapes, it is the preferred form. Since the surface/volume ratio of either type of chamber is inversely proportional to the cube root of the volume, it decreases as chamber size increases. Obviously, protuberances and extraneous apparatus or accessories such as fans and lights, within the chamber, which increase the surface/volume ratio should be avoided wherever possible.

Fans, often used as a means of promoting even distribution of agent in the chamber, have been found unnecessary in this laboratory. The air flow itself tends to produce a completely satisfactory distribution, even in the absence of mixing bowls or other premixing devices, as shown by the following data taken from work of Wells.[12]

*Cotoid, Lithgow Corporation, Chicago, Ill.

A concentration of chlorine was established in a 629 liter cubic chamber operated at an air flow of 500 liters per minute. Samples were taken from nine locations, identified by number, within the chamber. Sampling point 1 was the center of the chamber, while the other eight points were the eight corners. A series of experiments was performed in which samples were drawn simultaneously from point 1 and one other point, after equilibration. Concentrations were calculated from the results of iodometric analysis. It was found that the concentration in the corners was the same as that in the center of the chamber for any given sampling time. The results are given in Table IV.

TABLE IV. DISTRIBUTION OF CHLORINE IN CONSTANT FLOW CHAMBER

RUN NO.	SAMPLING POINTS	CONCENTRATION CHLORINE FOUND (MILLIGRAMS PER LITER)
1	1	0.97
	2	0.96
2	1	0.94
	2	0.94
3	1	0.97
	3	0.98
4	1	0.98
	3	0.97
5	1	0.97
	4	0.97
6	1	0.98
	4	0.97
7	1	0.97
	5	0.97
8	1	0.98
	5	0.98
9	1	0.97
	6	0.97
10	1	0.98
	6	0.98
11	1	0.97
	7	0.97
12	1	0.97
	7	0.97
13	1	0.97
	8	0.97
14	1	0.97
	8	0.97
15	1	0.97
	9	0.96
16	1	0.97
	9	0.97

RELATION OF DOOR SIZE TO AIR INLET

Concentration losses may occur if the chamber air inlet is too small in relation to the size of the door.

When the door of a gassing chamber is opened, air enters through the door as well as through the inlet. The proportion of the total air entering the chamber through these openings is governed by their proportional cross sectional areas and may be calculated.

One of the small animal chambers in this laboratory has a circular air inlet 2 inches in diameter (cross-sectional area = 3.1 square inches) and a square door measuring 7 by 7 inches (cross-sectional area = 49 square inches). When

the door is opened for inserting or withdrawing animals, 6 per cent of the chamber air flow enters the inlet, and 94 per cent enters the door opening. This particular chamber is usually operated at an air flow of 250 liters per minute. Thus, during the time the door is open, 235 liters per minute enter the door and 15 liters per minute enter the inlet. If an agent is being vaporized by means of air or inert gas, or is being dispersed in the gaseous state, the agent flow is limited to 15 liters per minute. Any flow in excess of that is not drawn into the chamber but is blown back into the room.

Although in actual operation the door of the chamber remains open only a brief period of time, this factor has an important influence on chamber behavior. Since the time required to restore the concentration to its previous level is the same as that for the original equilibration, a significant decrease in chamber concentration may cause serious errors for short exposure periods.

To promote turbulence and good mixing in the chamber, the agent inlet should be small enough to insure a rather high rate of linear flow. This limits the door size to a maximum cross sectional area determined by the desired agent flow and the chamber air flow. With higher rates of air flow, larger doors may be permitted, but the ease of setting up concentrations is thereby decreased because of the attendant necessary increase in rate of agent volatilization.

EFFECT OF ANIMALS OR SUBJECTS

Animals or subjects within a chamber have an effect upon the concentration because of the additional surface presented. This is demonstrated by the following experiments.

Clothed and naked men were exposed to various established concentrations of dichlorodiethyl sulfide vapor in a 20,000 liter chamber operated at an air flow of 3,600 liters per minute. The entry of the men into the chamber caused an almost immediate drop in the concentration which then remained for the duration of the exposure (from 10 to 120 minutes) at its new lowered level. The concentration drop was much more pronounced with clothed men than with naked men because of differences in the nature and quantity of the added surface. The data are presented in Table V.

TABLE V. CONCENTRATION DROP DUE TO SURFACE OF SUBJECTS

DRESS	NUMBER OF SUBJECTS	NUMBER OF EXPERIMENTS	AVERAGE CONCENTRATION DROP (PER CENT)	CONCENTRATION DROP PER SUBJECT (PER CENT)
Clothed	4	10	22.3	5.6
Clothed	5	29	26.7	5.3
Naked	5	13	8.0	1.6
Naked	8	10	11.8	1.5

TABLE VI. CONCENTRATION DROP DUE TO SURFACE OF ANIMALS

NUMBER OF MICE	NUMBER OF EXPERIMENTS	AVERAGE CONCENTRATION DROP (PER CENT)	CONCENTRATION DROP PER MOUSE (PER CENT)
20	26	17	0.9
10	2	10	1.0

In another series of experiments, placing mice in a 386 liter chamber operated at an air flow of 250 liters per minute, produced a drop in the previously established chamber concentration of chlorovinyl dichlorarsine. The results are shown in Table VI.

To minimize surface effects due to animals, the volume of the chamber should be large compared with the volume of the animals exposed. It has been found in this laboratory that excessive concentration lowerings occur when the volume of the animals is more than 5 per cent of the chamber volume.

The rate at which animals or subjects enter the chamber may also cause a drop in the concentration. The volume of animal or subject entering per unit time must be less than the volume of air entering the door, if displacement of chamber air into the laboratory is to be avoided.

Some examples will illustrate this point. In a large chamber used in this laboratory for human exposures, at an air flow of 4,000 liters per minute, 98.3 per cent or 3,932 liters per minute enter through the door when it is opened. This is equivalent to 65.5 liters per second. An average man, having a volume of about 70 liters, would therefore enter in not less than 1.07 seconds. In usual practice, about 1.5 seconds are allowed for each man entering the chamber.

A smaller chamber used for exposures of small animals is usually operated at an air flow of 250 liters per minute. When the door is opened, 4 liters per second enter the door. Twenty mice, occupying a volume under 0.5 liter, can be inserted in as quickly as 0.125 second without displacing the air. However, six rabbits, with a volume of about 12 to 15 liters, must be introduced over a period of from three to four seconds.

DISCUSSION

Serious errors in gassing experiments may be made if exposures are initiated before equilibration. Probably the greatest contributor to such errors is a misinterpretation of the term "air change." An "air change" is said to occur when a volume of air equal to the volume of the chamber has passed through the chamber. However, one "air change" does not completely renew the chaimber air, but, from Equation 2, should change the concentration within by only 63.2 per cent. From Equation 3 it is obvious that the time for one "air change" must be multiplied by a factor of 4.605 before even 99 per cent of the expected change can be accomplished. The term "air change" is therefore misleading, especially to the uninitiated, and should be eliminated from gassing terminology.

It is hoped that this exposition will be of help to those who have noted apparently irregular behavior in gassing chambers. A knowledge of the principles previously discussed should clarify many of these anomalies. The way in which concentrations build up has been discussed, and emphasis is to be placed upon the importance of the equilibration time. However, the effects of other factors on concentrations after equilibration cannot be neglected and may cause considerable deviations from calculated results. It is therefore necessary that regular analyses of chamber concentrations be made for the constant information of the operator.

SUMMARY

The principles discussed in this paper may be summarized as follows:

1. The *air flow* through the chamber influences:

(a) The time for equilibration to 99 per cent of theoretically constant concentration.

(b) The concentration which can be established for any given agent.

(c) The speed at which animals or subjects may be inserted.

2. The *size* of the chamber influences:

(a) The time for equilibration.

(b) The loss in concentration due to surface effects.

(c) The number of animals or subjects which can be exposed at one time.

3. The *character and quantity of the interior surface* of the chamber influence the loss in concentration due to surface effects.

4. The *shape* of the chamber influences the surface/volume relationship, and thus the loss in concentration due to surface effects.

5. The *relative areas of air inlet and door opening* influence the maximum rate of agent dispersal.

6. The *number and size of the animals* influence:

(a) The rate of insertion of animals into the chamber.

(b) The loss in concentration due to surface effects.

REFERENCES

1. Marshall, E. K., Jr., and Kolls, A. C.: An Apparatus for the Administration of Gases and Vapors to Animals, J. Pharmacol. & Exper. Therap. **12**: 385, 1919.
2. Fries, A. A., and West, C. J.: Chemical Warfare, New York, 1921, McGraw-Hill Book Company, Inc., pp. 353-355.
3. Drinker, P.: Laboratories of Ventilation and Illumination, Harvard School of Public Health, Boston, J. Industrial Hygiene **6**: 57, 1924.
4. Walton, D. C., and Jones, C. A: A Gassing Chamber for Short Exposures, J. LAB. & CLIN. MED. **11**: 580, 1926.
5. Sayers, R. R., Yant, W. P., Waite, C. P., and Patty, F. A.: Acute Response of Guinea Pigs to Vapors of Some New Commercial Organic Compounds. I. Ethylene Dichloride, Pub. Health Rep. **45**: 225, 1930.
6. Yant, W. P., Schrenk, H. H., and Sayers, R. R.: Methanol Antifreeze and Methanol Poisoning, Indust. & Engin. Chem. **23**: 551, 1931.
7. Dudley, H. C., and Miller, J. W.: Toxicology of Selenium. IV. Effects of Exposure to Hydrogen Selenide, Pub. Health Rep. **52**: 1217, 1937.
8. Dudley, H. C., and Neal, P. A.: Toxicology of Acrylonitrile I. A Study of the Acute Toxicity, J. Indust. Hyg. & Toxicol. **24**: 27, 1942.
9. Dudley, H. C., Sweeney, T. R., and Miller, J. W.: Toxicology of Acrylonitrile. II. Studies of Effects of Daily Inhalation, J. Indust. Hyg. & Toxicol. **24**: 255, 1942.
10. Miller, A. T.: A Respiratory Chamber for Chronic Exposure to Gases, J. LAB. & CLIN. MED. **28**: 1854, 1943.
11. Jones, G. W., Berger, L. B., and Holbrook, W. F.: Carbon Monoxide Hazards From House Heaters Burning Natural Gas, Bureau of Mines Technical Paper No. 337, 1923.
12. Wells, W. J. H. B.: Personal communication.

THE SIMPLE ESTIMATION OF BLOOD KETONES IN DIABETIC ACIDOSIS

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IT CANNOT be too strongly emphasized that diabetic acidosis demands early diagnosis and prompt treatment. Occasions arise when clinical appraisal of the patient is inadequate for positive diagnosis. Emergency determinations of blood glucose and carbon dioxide combining power are sometimes employed. A high blood sugar level, however, is not diagnostic of acidosis, and a reliable determination of CO_2 is not always obtainable in an emergency. Because there is a parallel between the blood ketone level and the severity of diabetic acidosis, a simple clinical method for the estimation of blood ketones would be of distinct value.

Routinely, the urine is tested for acetone and diacetic acid. Several authors have suggested the use of a dry powder for the qualitative detection of these compounds.[1-3] A drop of urine added to the powder gives a prompt and distinctive purple color when acetone is present.* There are a number of diabetic patients whose disease is severe and who frequently show large amounts of sugar in the urine. Under the influence of a slight infection, these patients may rapidly develop a diabetic acidosis. Since the early detection of ketonuria, when followed by prompt therapy, will do much to prevent the onset of clinical acidosis, we have found it worth while to provide the patients with more severe diabetes in our clinic with this powder. The test is simple and inexpensive and need only be used when the routine test for sugar indicates excessive glycosuria.

We have studied the application of this test to the determination of blood ketones, and it has been found that the same color reaction will appear when serum of a high ketone content is added to the powder. The minimal blood level of total acetone bodies giving a definitely positive test is approximately 10 mg. per 100 c.c. as determined by the method of Shipley and Long.[4] Thus, the test can quickly be made quantitative by the successive dilution of serum with distilled water. The last dilution to give a positive reaction, when multiplied by ten, will give the blood ketone level within an accuracy of plus or minus 10 mg. per 100 c.c. For example, if the undiluted serum has been shown to give a positive test, then 1 c.c. is mixed with an equal amount of water. If a positive reaction is still present, additional portions of water are added until the test becomes negative. In a given sample, the last positive dilution might contain 1 c.c. of serum and 6 c.c. of water. This would represent a dilution of

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*Our own formula for the powder has been:

Sodium nitroprusside, 1 Gm. (very finely ground)
Ammonium sulfate, 20 Gm.
Anhydrous sodium carbonate, 20 Gm.

The three ingredients are mixed completely but are not ground together. The compound should be kept dry at all times. Stability has been shown to be complete for over one year.

The test is performed by placing a small pinch of the powder, 5 mm. in diameter, on a white filter paper. One drop of urine or serum gives a prompt violet color reaction which is absorbed into the paper and which may last for several hours.

A powder of somewhat similar composition is available commercially.

1 part in 7, and the result would be approximately 70 mg. per 100 c.c. If only one drop is removed after each succeeding dilution, the error introduced by withdrawal of the test sample will be less than 5 per cent.

The value and relative accuracy of this simple test have been shown in the diagnosis and treatment of diabetic coma at Lakeside Hospital. It has been the practice recently to follow the blood ketone levels during active therapy in diabetic acidosis. In some twenty-five instances, the values of the simple spot test have been shown to correspond to the more accurate simultaneous micro determinations with an error seldom exceeding 10 mg. per 100 c.c. The range covered has been from 0 to 100 mg. per 100 c.c. It is true that the micro test determines total ketone bodies while the test with the powder measures only acetone and diacetic acid, yet the numerical values have been valid in the range mentioned. This accuracy has been so consistent that it has become routine to use the test when there is any doubt as to the severity of ketosis. By virtue of its rapidity and simplicity, it has proved more valuable than the emergency determinations of CO_2 combining power in providing immediate laboratory confirmation.

In the diagnosis and treatment of diabetic acidosis, the following seems to be worth emphasis. With a typical history and findings, diagnosis presents no problem. Often, however, the history is not typical. More important, the presence or absence of acetone in the urine may not indicate the actual blood ketone level. It has been pointed out that in renal failure, ketonuria may be minimal in spite of severe ketonemia. Furthermore, in patients without renal failure but with diminished kidney function, the threshold for ketone excretion may be abnormally high. Thus, the previously mentioned test which measures blood ketone levels will be considerably more useful than a test for acetone in the urine. It has also been valuable in quickly separating true diabetic acidosis from those cases in which a poorly controlled diabetes has been accompanied by a surgical abdomen, by vomiting, or by coma from other causes and in which blood ketones have not been high. In these latter cases, the vigorous therapy demanded in true diabetic acidosis is not needed, and the necessary surgical therapy can proceed promptly. Ketone levels in these cases seldom exceed 20 mg. per 100 c.c., while those of diabetic acidosis are invariably over 50 mg. per 100 c.c.

SUMMARY

Acetone bodies in serum may be estimated within an error usually not exceeding 10 mg. per cent by the use of a simple technique employing a nitroprusside powder. The method is particularly useful for the prompt differentiation of minimal ketonuria due to minimal ketonemia, as contrasted with cases of a high renal threshold resulting from depressed renal function in which minimal ketonuria may be associated with severe ketonemia.

REFERENCES

1. Laughlen, G. F.: A Convenient Test for Acetone, Canad. J. M. Technol. 5: 3, 1943.
2. Ingram, John: A New Test for Acetone, Brit. M. J. 1: 512, 1944.
3. Rabinowitch, I. M.: A Simple Test for Acetone in the Urine, Canad. M. A. J. 52: 602, 1945.
4. Shipley, R. A., and Long, C. N. H.: Studies on the Ketogenic Activity of the Anterior Pituitary, Biochem. J. 32: 2242, 1938.

BOOK REVIEWS

Die Grundlagen unserer Ernährung und unseres Stoffwechsels. By Emil Abderhalden, Professor an der Universität Zürich. Medizinscher Verlag Hans Huber, Bern, 1946. Paper, 202 pages. Price Fr. 8.50.

Zur Physio-Pathologie Der Niere. By Dozent Dr. med. Otto Spühler, Oberarzt der Med. Universitäts-Poliklinik Zürich, mit einem Vorwort von Prof. Dr. med. P. H. Rossier. Medizinischer Verlag Hans Huber, Bern, 1946. 248 pages with 22 illustrations. Cloth binding, Fr. 17.50; cardboard binding, Fr. 15.60.

ANNOUNCEMENT

The first session of the Pathology Study Section of the National Institute of Health's Research Grants Division was held at the Institute in Bethesda, Md., on Aug. 16, 1946.

The Pathology Study Section, which is one of more than twenty groups of outstanding consultants advising in the whole field of medical research, is comprised of the following men: Dr. Paul Cannon, Chairman, University of Chicago, Chicago, Ill.; Dr. W. A. DeMonbreun, Vanderbilt University, Nashville, Tenn.; Dr. W. H. Feldman, Mayo Foundation, Rochester, Minn.; Dr. W. D. Forbus, Duke University, Durham, N. C.; Dr. H. Goldblatt, Cedars of Lebanon Hospital, Los Angeles, Calif.; Dr. J. S. McCartney, University of Minnesota, Minneapolis, Minn.; Dr. A. R. Moritz, Harvard University, Boston, Mass.; Dr. A. Rich, Johns Hopkins University, Baltimore, Md.; Dr. J. F. Rinehart, University of California, San Francisco, Calif.; Dr. H. P. Smith, Columbia University, New York, N. Y.; representatives of the Surgeons General of the United States Army and United States Navy, members of the Medical Department of the Veterans' Administration, and Dr. R. D. Lillie, National Institute of Health, Bethesda, Md., as Executive Secretary.

A number of projects were considered and recommendations made to the National Advisory Health Council for final action. The Section members agreed unanimously that individually they would promote necessary research in the field of pathology by private suggestions but would not make any collective attempts to impose research projects on investigators or institutions. They also approved the attitude of the Public Health Service that the scientific freedom of the research investigators must not be restricted in any way.

Application forms for Grants-in-Aid may be obtained from the Chief, Research Grants Division, National Institute of Health, Bethesda 14, Md., and for prompt action should be filed well in advance of the quarterly meetings of the Study Section concerned.

The next meeting of the Pathology Study Section was scheduled for an early date in November, 1946.